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Characterization of an immunizing antigen with endotoxin-like properties isolated from Pasteurella multocida

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Characterization of an immunizing antigen with endotoxin-like properties isolated from *Pasteurella multocida*

by

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TABLE OF CONTENTS

INTRODUCTION

Fowl cholera (Avian Pasteurellosis) is a major infectious disease affecting many species of birds. The causative agent is Pasteurella multocida, a gram-negative bacterium. This disease and organism are of historical interest due to the pioneering achievements of Louis Pasteur in successfully immunizing chickens through the vaccination with attenuated strains. Despite the success of Pasteur, the attenuated state of the organism has not been uniform enough for the consistent use of a safe attenuated vaccine strain.

In recent years there has been widespread use of both commercially and experimentally prepared killed bacterins to immunize fowl. However, the use of these vaccines has not consistently protected fowl from infection. Although the reasons for this are not known, it appears that the answers to two fundamental questions will aid in the development of effective vaccines. These questions are: first, how many different immunogenic types of Pasteurella multocida occur in natural infections of fowl and second, what is the nature of the immunogenic antigen or antigens of the organism?

The purpose of this study was to attempt to answer the last of the above questions. More specifically, the intent of this investigation has been to isolate and characterize the chemical, physical and biological properties of an immunogenic substance found to be easily extracted from intact

P. multocida. Both encapsulated, virulent and nonencapsulated, avirulent forms of P. multocida have been described by Heddleston (1962) and both forms of the organisms have been found to be highly immunogenic in chickens when used as killed bacterins. The substance characterized in this investigation was isolated from saline extracts of the avirulent non-encapsulated P. multocida strain P-1059 Gray. Since this organism did not have a capsule, it was felt it would be easier to isolate and characterize the immunogenic substance. The P-1059 strain (originally a turkey isolate) has been described by Heddleston and co-workers (1962, 1966) and is considered to represent the most prevalent P. multocida immunogenic type found in field cases of fowl cholera.

REVIEW OF LITERATURE

Although few extensive studies of immunogenic[1] macromolecules isolated from Pasteurella multocida have been reported, several investigators have tried to immunize animals with various cell fractions, toxins, and antigens obtained from P. multocida. Many of the immunity studies have also been carried using attenuated or killed organisms. The review of all the literature pertaining to immunity studies with P. multocida was not attempted but rather a review was made of those articles which describe or attempt to describe the biochemical nature of the immunogenic antigens. An extensive review of the serotyping of P. multocida is not included here. However, reference is made to various serotyping reports which include immunity studies or attempt to relate the various serotyping systems to immunological types. The review of the literature on the antigens and toxins of P. multocida has been written using an historical sequence as most of the published reports depend on previous studies rather than being the description of isolated experiments.

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[1]Immunogenic - this term will be used throughout this dissertation to denote the ability of an injected substance to actively protect a host upon subsequent exposure to a virulent organism.

Following the review of P. multocida toxins and immunogens is a brief review of the toxins of other bacteria with particular emphasis on the endotoxins or lipopolysaccharide complexes of gram-negative organisms. There is a vast literature on endotoxins but references are made to selected articles which it is felt pertain most directly to the P. multocida immunogenic complex described in this dissertation.

The Antigens and Toxins of Pasteurella multocida

One of the earliest immunity studies with P. multocida was made by Pasteur. In these now classical studies Pasteur (1880b) found that chickens could be successfully immunized to fowl cholera with attenuated strains of P. multocida. In addition Pasteur (1880a) reported the presence of a "narcotic" or toxin in culture filtrates which produced drowsiness when injected into chickens. Shortly thereafter Salmon (1881-1882) repeated the work of Pasteur and also found a toxic substance in the culture filtrates. He tried to immunize chickens against fowl cholera with these heat concentrated (boiling water bath) culture filtrates but a series of six injections over a period of approximately six weeks failed to protect the chickens when challenged with P. multocida. Several subsequent investigations found neither a toxin nor immunogen in the culture filtrates.

Many years later Priestley (1936) performed immunity studies with P. multocida (then called Pasteurella septica) and proposed that the organism contained both a heat-stable somatic antigen and a heat-labile (56°C, 60 min) capsular antigen. The capsular antigen was associated only with the virulent organism. He found that organisms killed by low temperatures (56°C, 30 min) retained their capsules and immunized against infection while virulent organisms killed by high temperatures 100°C, 60 min) lost their capsules and their ability to immunize. A virulent organism (i.e. non-encapsulated) failed to immunize. All bacterins were injected three times at six-day intervals. In 1938, Pirosky (1938) reported the use of the Boivin method to extract and isolate four different toxic glycolipids from smooth and rough strains of P. multocida. He found that rabbit antiserum to these toxins would passively protect mice when they were subsequently injected with toxins but studies of active immunity to infection were not reported. A suggestion was made by Kyaw (1944) that a toxin was produced during in vivo growth of P. multocida and that this toxin was responsible for the pathogenic effects observed when the organisms were grown on the chorio-allantoic membranes of chick embryos. The embryos appeared to die from a progressive toxaemia.

Little and Lyon (1943) found that rabbit and horse antiserum to various formalin killed P. multocida isolates would passively protect mice to challenge with the virulent organism. This protective ability was used to type the P. multocida into three serotypes designated 1, 2, and 3. In addition they found the serum could be used in a slide agglutination test. Later Roberts (1947) also used the ability of antiserum prepared in rabbits with heat killed organisms to passively protect mice against infection to classify the P. multocida into four immunological types. The types were designated types I, II, and III, and IV and antiserum to the type I was found to give specific passive protection while there was some cross protection in the other groups.

The isolation of an immunogenic "capsular polysaccharide" was reported by Carter and co-workers (1952, 1953, and 1955). This "polysaccharide" was isolated by heating various capsulated P. multocida (56°C, 60 min) and precipitating the "polysaccharide" from solution with three volumes of alcohol. It was then used to prepare specific antisera in rabbits. Using the precipition test Carter and Annau (1953) classified the P. multocida into four types designated A, B, C, and D. The specific "polysaccharide" when isolated from fluorescent virulent type B organisms was found to be specifically immunogenic for mice when the mice were injected three times with 50 micrograms. However, "polysaccharide" from mucoid

strains (i.e. strains containing a large capsule of hylauronic acid) failed to immunize mice under these conditions. The "polysaccharide" isolated by Carter from type B contained approximately 5% nitrogen, 36% reducing substance and 2-4.6% phosphorus and most probably contained protein and/or nucleic acid in addition to polysaccharide.

A comparison of the virulence of encapsulated and non-encapsulated serotypes (Little and Lyon's types 1, 2, and 3) of P. multocida was made by Yaw et al. (1956). It was found that whereas encapsulated organisms of the three serotypes were virulent for mice, chickens were only susceptible to type 1. In addition immunization with encapsulated types 2 and 3 did not protect chickens challenged with type 1 demonstrating the immunological specificity of the types. Subsequently Yaw and Kakavas (1957) demonstrated the difference in response of chickens and mice to the somatic and capsular substances. They found that a single injection of the nonencapsulated bacteria or three injections of capsular polysaccharide protected 90-100% of the young chickens. However, in mice two and three injections of the nonencapsulated organism only protected 0 and 50% of the mice respectfully while two and three injections of the capsule substance protected 90 and 100% of the mice. In addition these investigators suggested that there might be different somatic antigens in the different serological types as a serotype three vaccine failed to protect against a type 1 challenge. A similar observation

was made by MacLennan and Rondle (1957) when they found that cross absorption studies of an antiserum of one type with the organisms of another type revealed a type specific antigen which gave a serological reaction of identity with a phenol extracted lipopolysaccharide when the two were examined in Ouchterlony gel diffusion plates.

A somewhat more thorough investigation of the antigens of bovine Type 1 P. multocida has been reported (Knox and Bain, 1960, Bain and Knox, 1961). Knox and Bain reported that isoelectric precipitation at pH 3.8 of saline extracts (2.5% w/w NaCl) resulted in precipitation of lipopolysaccharide and protein and that the supernatant contained a separate polysaccharide easily distinguished by the presence of a ketose, which the authors thought might be fructose, and the absence of a heptose. This "polysaccharide" provided active immunity in mice when crude preparations (6.8% nitrogen) were injected (one 40 µg injection, followed by challenge three weeks later) but "polysaccharide" preparations in which the protein was removed by trypsin were not effective. The lipopolysaccharide was isolated by Bain and Knox (1961) from this same organism using the phenol procedure. It was found to contain a heptose, glucose, galactose, and glucosamine. However, the preparation was not toxic at 1 mg. levels in mice. The lipopolysaccharide from a related strain was toxic. Neither lipopolysaccharide would immunize to any significant

extent mice subsequently challenged with homologous virulent organisms. Single injections of 20-200 µg protected 20% of mice challenged three weeks later. Multiple injections of 40 µg still gave a maximum of 20% protection.

The fact that there are at least two immunogenic types of P. multocida associated with fowl cholera was reported by Heddleston (1962). Immunization with one formalin killed strain (X-73, a chicken isolate) did not significantly protect chickens which were subsequently challenged with another strain (P-1059, a turkey isolate) and the reciprocal test also showed little cross-protection. Homologous immunizations and challenges resulted in 90% protection with strain P-1059 and 100% protection with X-73. Chickens inoculated with a bivalent vaccine were highly resistant to infection with either strain. The X-73 strain appeared to be more virulent as it killed both 16 week old and 45 week old chickens while the P-1059 strain killed the 45 week old chickens but not those 16 weeks old. The P-1059 strain did kill 16 week old turkeys. The X-73 strain was classified as serotype 1 and the P-1059 strain as serotype 3 using Little and Lyon's classifications.

Due to the observation that the somatic antigens appeared to be important in providing immunity at least in fowl, a serological typing system was devised by Namioka and coworkers (Namioka and Murata, 1964, Murata et al., 1964) which was based on the (0) somatic groups. The (0) antigen was

produced by treating the organisms with 1 N HCl. Using an agglutination test with various (O) group absorbed sera Namioka and co-workers classified the P. multocida into ten serotypes based on the somatic antigen. A large number (118) of strains were typed by their somatic antigen and Carter's capsular antigen system. Heddleston's X-73 and P-1059 strains were typed 5:A and 8:A respectively. All the fowl cholera strains were found in serotypes 5:A, 8:A, or 9:A.

The isolation from a P. multocida African bovine strain of a lipopolysaccharide which is part of the somatic antigen was reported by Perreau and Petit (1963) using the Westphal phenol procedure. This strain of P. multocida was classified as Type E using Carter's system. The isolated lipopolysaccharide would not directly immunize mice to infection with Type E; however, when it was adsorbed to erythrocytes and injected into rabbits it would induce antibodies that would passively immunize mice to Type E. The lipopolysaccharides from Type B and Type E were shown to be serologically distinct and to cause pyrogenicity and leucopenia in rabbits.

The fact that the presence of a capsule on P. multocida was most often correlated with virulence but not with the presence of immunogenic antigens was pointed out by Heddleston et al. (1964) when it was found that virulent capsulated cultures (a fluorescent - iridescent colony type) would

dissociate into a less virulent blue colony type and then to avirulent nonencapsulated gray colonies. These avirulent gray colonies still contained an immunogenic antigen.

The isolation of particulate toxic antigens from avian and bovine isolates of P. multocida which will actively immunize either mice, chickens, turkeys, rabbits, or cattle has been reported in a series of papers by Heddleston, Rebers, and co-workers (Rebers et al., 1965; Heddleston et al., 1966; Heddleston et al., 1967; Rebers et al., 1967; Heddleston and Rebers, 1968; Heddleston and Rebers, 1969; Ganfield et al., 1970). These investigations have shown that high-speed centrifugation (105,000 xg, 2 hrs.) of culture filtrates of P. multocida sediments a substance which is toxic, actively immunizes animals to specific challenge and contains the chemical components found in endotoxins. In addition the immunological responses of these substances correlated with immunity studies carried out with formalin killed organism vaccines. The publication of Heddleston et al. (1966) demonstrates that particulate antigens can be isolated from two different immunogenic types of P. multocida and that these preparations were serologically and immunologically distinct. When electron micrographs of the particulate antigens isolated from X-73 gray were examined, the antigenic preparations were found to contain a large number of globular particles. Chemical analysis revealed that the preparations contained

substantial amounts of protein and carbohydrate. They also gave a positive reaction with Sudan Black indicating the presence of lipid.

Two recent publications (Brown et al., 1970, Srivastava et al., 1970) have reported attempts to isolate and study immunogenic cell functions and culture filtrates of P. multocida P-1059 fluorescent. This is the same immunogenic type of P. multocida used for the experiments described in this dissertation. The culture was obtained from K. Heddleston of this laboratory (National Animal Disease Laboratory). Brown et al., 1970 reported that P. multocida P-1059 fluorescent culture filtrate saline solutions when injected into turkeys at 8 weeks and again at 15 weeks protected 77% of birds which were challenged at 20 weeks of age. Commercial bacterins gave inconsistent immunity results varying from 27% protection in one experiment to 66% in another. A similar type of investigation was reported by Srivastava et al. (1970) in which mice were immunized with components of P. multocida P-1059 fluorescent. Three components (cell wall, cytoplasm, and culture filtrate) gave some protection to mice which were immunized 45 days prior to homologous challenge. The culture filtrate was fractionated by Sephadex G-50 column chromatography and most of the immunogenicity was found in the first component eluted from the column. The authors did not feel the culture filtrate fraction was the same particulate

immunogen described by Heddleston et al. (1966) due to the low heptose and 2-keto-3-deoxyoctulosonic acid content.

Bacterial Toxins

Historically the bacterial toxins have been classified by their cellular location. The term exotoxin is used for those toxins which are liberated naturally from the organism and are therefore found in the extracellular medium. Those toxins thought to occur in an intracellular location have been designated endotoxins. This terminology, although still used, is in many ways inaccurate and perhaps should be replaced. This fact is most obvious when recent descriptions of "free endotoxin" are considered.

The bacterial exotoxins have been generally isolated from cultures of gram-positive microorganisms and chemical studies have demonstrated that they are proteins. Typical protein exotoxins include a number of toxins produced by the germs Clostridia, as well as the toxins of Corynebacterium diphteriae, Stapholococcus ameuis and Streptococcus pyogenes. The protein exotoxins are some of the most toxic substances known and the most notable of these are the botulinium, tetanus, and diptheria toxins. These toxins are commonly detoxified by the use of heat or formaldehyde and subsequently used for immunizations. In some cases the exotoxins have been found to occur intracellularly in the bacteria as well as in the

extracellular fluid. An excellent review of the protein microbial toxins can be found in the first three volumes of a book series entitled "Microbial Toxins," (Ajl, Kadis, and Montie, eds. 1970). Future volumes in this series are planned which include the review of endotoxins.

The true endotoxins based on the definition given previously are perhaps best represented by the protein endotoxins which have been isolated from disrupted gram-negative bacteria. Endotoxins of this type include the toxins of Bordetella pertussis, Vibrio cholera, Shigella dysenteriae, and Pasteruella pestis. The best characterized of these toxins are the dysentery neurotoxins (van Heyningen and Gladstone, 1953) and the murine plague toxins (Montie et al., 1966). These toxins do not appear to be attached to the cell wall and immunization with the corresponding toxoids does not protect against infection with the organism. An extensive review of the literature of the plague toxin has appeared recently (Montie and Ajl, 1970). The literature on the other protein endotoxins has been reviewed recently by Raynaud and Alouf (1970).

The Enterobacteriaceae and many other gram-negative bacteria contain a toxic lipopolysaccharide-protein-lipid complex associated with their cell wall which is most commonly referred to as endotoxin. Although the term endotoxin is most frequently associated with these complexes, this term has been

shown to be inaccurate and misleading. It has been shown recently that these complexes are actually located on the surface of the cell wall and frequently extend from the surface rather than being located intracellularly (Mergenhagen et al., 1966; Shands et al., 1967). Earlier studies of Boivin and Mesrobeanu (1935) support this surface location as they found that these toxic complexes could be extracted from intact bacteria. In a following part of this review which covers "free" endotoxins it is evident that in many cases these complexes are actually excreted from the organisms so they may be described as "extracellular" endotoxins.

Since the lipopolysaccharide complexes display a large number of biological activities, there is an overwhelming number of publications pertaining to their isolation and activities. It is not possible nor pertinent to try to review this extensive literature here. The references which will be mentioned in the following section are only the more basic articles which describe the various methods of isolation of these toxins. Several extensive reviews and symposiums on endotoxins have been published and reference will be made to the more recent of these.

The endotoxins of the Enterobacteriaceae and various other gram-negative bacteria have been isolated from the intact bacteria or cell walls in most cases by fairly drastic methods. Some of these procedures include the trichloroacetic

acid extraction at 4°C (Boivin and Mesrobeanu, 1933), aqueous phenol extraction at 68°C (Westphal et al., 1952), aqueous ether extraction at 6-12°C (Ribi et al., 1959) water extraction at 80° (Roberts, 1966) and extraction with 1M NaCl containing 0.1M sodium citrate (Raynaud et al., 1964). In each case the chemical, physical, and often the biological properties of the recovered endotoxin differ considerably. Because of the drastic extraction methods it is very difficult to say how close the extracted material resembles the original complex.

Perhaps the most common of these drastic methods of extraction is the aqueous phenol procedure of Westphal. This procedure is similar in many respects to the method used to separate proteins and nucleic acids. The extracted proteins go into the phenol phase while the lipopolysaccharides remain in the aqueous phase. This procedure has been most commonly used with various strains of Salmonella and E. coli. The extracted lipopolysaccharides have been studied extensively including their chemical physical, and biological properties. Perhaps the most significant observation of these preparations is that the chemical composition of the polysaccharide chains in particular the terminal sugars can be correlated with specific immunological reactions with antiserum. The Kaufman-White scheme of serotyping of the Salmonella genus and the serotypes of E. coli have been correlated with their respective chemotypes. An excellent and extensive review of this subject

has been published recently by Lüderitz et al. (1966). Lipopolysaccharides as isolated by the phenol procedure are generally recognized as being poor antigens.

The other relatively drastic methods of extracting the lipopolysaccharide (LPS) complexes differ primarily in the relative amounts of protein, peptide or lipid which are associated with the LPS. The Boivin method of isolation usually results in the isolation of a complex which contains LPS combined with a large amount of protein or peptide. This antigenic material is often called the "Boivin" or somatic "O" antigen when extracted from smooth variants of gram-negative bacteria. The Boivin antigens appear to have all the biological activities of LPS but in addition are capable of stimulating the appearance of specific agglutinating antibodies in various animals. The protein or polypeptide moiety of the Boivin antigen is thought to be chiefly responsible for this immunogenicity while the LPS portion of the complex accounts for the toxicity. However, there are a few instances where the protein moiety is also thought to be toxic.

An example of this is the description by Mesrobeanu et al. (1966) of the thermolabile neurotoxins isolated from Salmonella typhinurium which are thought to be the same as the protein or peptide moiety of the Boivin type antigens. In addition to being toxic these proteins have been found to be antigenic and in many cases bacteriocidal. The neurotoxins

cross react with antisera to the Boivin type antigens but not with antisera to the "purified" LPS. In a similar type of investigation Jenkin and Rowley (1959) found that the Boivin antigens of Vibrio cholerae contained a protein which was toxic. In their opinion it could account for the major toxic effects of the organism. The protein contained in Pseudomonas aeruginosa endotoxins was studied by Homma and Suzuki (1966) and it was found to have a pyocinic (i.e. inhibited the attachment of bacteriophage) activity. The amino acid composition of this protein was determined and found to differ from the composition of the bacterial mucopeptide layer. Antisera to this protein did not cross react with the P. aeruginosa phenol-extracted lipopolysaccharide.

An extensive review of the molecular and biological properties of the lipopolysaccharide and its complexes has been recently published by Nowotony (1969).

Extracellular or Free Endotoxins

In just the past few years another type of LPS endotoxin has been described. Although it had been known for some time that toxic substances were often found in the culture media of gram-negative organisms, their presence was generally assumed to result from bacterial autolysis. However, recent publications most notably those of Work and her collaborators have shown that gram-negative organisms such as E. coli actively

excrete a phospholipid-lipopolysaccharide-protein complex into the culture medium. This was most noticeable when lysine--requiring E. coli mutants lacking 2,6-diamino-pimelate decarboxylase were grown under lysine limiting conditions (Knox et al., 1967, Knox et al., 1966, Taylor et al., 1966).

The term "free endotoxin" was first used by Crutchley et al. (1967) to refer to a similar extracellular complex from E. coli grown under highly favorable conditions. This endotoxin was purified and analyzed by Marsh and Crutchley (1967). The amino acid and elemental analysis of this toxin showed it contained approximately 4.95% N, 10.06% P, 12.3% glucosamine and 14.1-17.7% amino acids. The structure of these free endotoxins was examined by Marsh and Walker (1968). They found a substantial number of rod-like particles when examined by negative staining in the electron microscope. These particles measured approximately 295 X 65 nm and were assumed to represent the toxic lipopolysaccharide-protein complex with a calculated molecular weight of approximately one million. Typical phenol or ether extracted endotoxins examined by these investigators were found to contain highly aggregated materials.

The investigations of Work and colleagues mentioned previously also included the chemical, biological, and structural characterizations. This work is also of particular note because of the many similarities between their E. coli extracellular LPS-protein-lipid complexes and the toxic and

immunogenic complex of Pasteurella multocida whose isolation and characterization is described in this dissertation.

The extracellular LPS complex isolated by Work and others and described by Knox et al. (1966) had approximately 2.3-3% N, 3.3% P, 10-11% heptose, 9-11% protein, and 23% extractable lipid. This analysis was interpreted to represent an excreted complex containing approximately 60% lipopolysaccharide, 26% extractable phospholipid, and 11% protein. The phospholipid was primarily phosphatidyl ethanolamine.

The biological testing of this complex and a phenol extracted lipopolysaccharide showed the LD_{50} in the chick embryo test to the 8.5 µg and 3.3 µg per embryo respectively. Similarly the complex was less toxic in mouse lethality tests then the "purified" lipopolysaccharide with LD_{50} values of 730 µg and 250 µg respectively.

Electron micrographs of the complete complex using negative staining revealed a mixture of spherical particles having diameters from 12 to 200 nm. Some rod shaped particles were also observed of variable lengths and having diameters of approximately 12 nm. The isolated complex closely resembled the wall blebs and extracellular globules that were shown to be excreted from the outer surface of E. coli in a prior publication (Knox et al., 1966).

A more recent study of the extracellular LPS-protein complexes of E. coli has been reported (Rothfield and Pearlman-Kothencz, 1969). This investigation dealt primarily with the

synthesis and isolation of the complex. Radioactive labels were used to follow the accumulation of excreted LPS, protein, and phospholipid. Lipopolysaccharide was labeled in E. coli mutants lacking UDP-galactose-4-epimerase through the use of ^{14}C galactose. In some experiments the phospholipid was labeled via U-^{14}C oleic acid and the proteins via growth in ^{14}C leucine. And in other experiments excreted material was doubly labeled with ^{3}H galactose and ^{14}C leucine. The use of labels made it possible to show that carbohydrate, protein, and lipid occurred in the same component when analyzed by sucrose density gradient centrifugations, gel filtration on Sephadex G-100, and in precipitin reactions. When protein synthesis was inhibited with chloramphenicol, the excreted complex had a lower protein content. This was also true when the cells were allowed to grow in conditions where essential amino acids were limited. The complex was found to contain phospholipid, lipopolysaccharide, and protein in a ratio of 3.4:2.4:1 by weight and to have a buoyant density of 1.27 g/cm^3 in sucrose density gradients. An important observation made as a result of these experiments is that the excreted membrane fragments may decline in their protein content as the cells reach a stationary phase where protein synthesis is limited. This observation would be important if one tried to isolate membrane fragments with relatively constant ratios of protein. The protein isolated by these investigators from the membrane

fragments appeared to be homogenous when subjected to acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

The isolation of a LPS-protein complex by warm water treatment (0.1 M Tris (trihydroxyaminomethane), pH 7.3, 48°C) of E. coli A was reported recently by Rogers (1971). It was found that the complex could be removed without the loss in viability of the organism and that the release of the complex was prevented by the presence of divalent cations. The complex was purified by DEAE cellulose chromatography and found to contain the LPS components (heptose, and 3-deoxyoctulosonate) and protein distributed in the same fractions. The protein amounted to 49.5% of the dry weight of the complex. The complex was dissociated by chromatography in the presence of EDTA (ethylenediamine tetracetic acid) or sodium deoxycholate. Quantitative amino acid analysis showed a full compliment of amino acids with aspartic acid, glutamic acid, alanine, glycine, leucine, and proline present in the highest amounts.

MATERIALS

The aqueous reagents and solutions used in this investigation were prepared using distilled, deionized water. The following organic solvents were re-distilled prior to use: ethyl alcohol, acetone, butanol, methanol, phenol, and chloroform.

Sources of Chemicals and Biochemicals

The chemicals used in these studies were obtained from the following sources: "Seakem" agarose was purchased from Bausch and Lomb, acrylamide from Eastman Organic, reagent grades sucrose and sodium chloride from Merck, reagent grade potassium bromide from Mallinkrodt, Sudan Black from Allied Chemical Corporation, Amido black 10B from E. Merck (Germany), Fat Red 7B from Keystone Aniline and Chemical Co., polyvinylpyrrolidone from General Aniline and Filter Corp., Arlacel A (mannide monooleate) from Atlas Powder Co., Bayol F (a light mineral oil) from Esso Standard Oil Co., Merthiolate (thimersol) from Eli Lilly, p-Anisidine - HCl grade III from Sigma Chemical Co.

The following biochemicals and biological materials were obtained from the following sources: Pronase (B grade) was purchased from Calbiochem, Glucostat and Galactostat from Worthington Biochemicals, α-D-glucoheptose and D-mannoheptose from General Biochemicals, bovine plasma albumin (an A grade,

crystalline Pentex, Inc. Product) from Calbiochem, E. coli 0111:B4 lipopolysaccharide (Westphal preparation) from Difco Laboratories, Blue Dextran, Sepharose 2B and Sepharose 4B from Pharmacia Fine Chemicals, Inc., Phosphatidyl ethanolamine (A grade) from Calbiochem, Phosphatidyl serine from General Biochemicals, and Tripalmitin from Eastman Organic.

A preparation of 80s porcine liver ribosomes was a generous gift of Marshal Phillips of the National Animal Disease Laboratory, USDA, Ames, Iowa.

Source of Animals

One week old Beltsville Small White Turkey poults were obtained from a closed NADL flock which is free of detectable antibodies to Pasteurella multocida. The mice used in immunity studies and toxicity studies were 16-18 gm female Swiss Webster mice obtained from either Simonsen Laboratories, St. Paul, Minnesota, or Carworth Farms, Portage, Michigan. Nine to ten day old chick embryos used in toxicity studies were obtained as fertile eggs from Larsen's Laboratory Eggs, Inc., Ft. Dodge, Iowa. The young, mature New Zealand rabbits were obtained from the NADL colony and were free of Pasteurella multocida antibodies as detected by gel precipitin methods.

Equipment

The following special equipment and materials were products of the following companies: Cellogel (gelatinized cellulose acetate) was obtained from Colab, Inc., Diaflo membranes and a model 400 filtration apparatus from the Amicon Company, Cellulose Casings from Union Carbide, glass fiber silica gel sheets (ITLC-SG) from Gelman Instruments Company, the Eastman Sandwich chamber developing apparatus from Distillation Products Industries, the Disc Electrophoresis apparatus from Canalco, "Oak Ridge" type polycarbonate centrifuge tubes from International Equipment Company, gel filtration columns and accessories from Pharmacia Fine Chemicals, Collodion Membranes and ultrafiltration apparatus from the Carl Schleicher and Schuell Co., 47 mm Membrane filters and a 100 ml stainless pressure filter holder from Millipore Corp.

METHODS

Bacteriological Methods

Preparation of media

The media used for the cultivation of the majority of the bacterial lots of P. multocida P-1059 Gray was a modification of the Dextrose Starch Agar medium described by Difco. In the modification gelatin and starch were omitted to reduce the chance of contamination of the particulate immunogenic preparations with medium components. The modified medium has been described by Rebers et al. (1967) and is composed of the following ingredients per liter of distilled water: 15 g of Proteose Peptone no. 3 (Difco), 2 g of glucose, 5 g of NaCl, 3 g of Na_2HPO_4 and 20 g of Ionagar no. 2 (Consolidated Laboratories, Inc.). The above ingredients were dissolved by heating in a boiling-water bath, the solution removed and allowed to cool to 60°C, and the pH was adjusted to 7.4. The medium was then added to Roux bottles and autoclaved for 20 minutes.

Cultivation of Pasteurella multocida

The type P-1059 Gray Pasteurella multocida (ATCC 15742) was grown and harvested by Mr. Kenneth Heddleston (National Animal Disease Laboratory, Ames, Iowa) or under his supervision. These methods have been described in detail by Heddleston and co-workers (Rebers et al., 1967).

The general method of cultivation involved the use of the Proteose Peptone no. 3 agar media in Roux bottles. The inoculated Roux bottles (usually 40-60) were incubated at 37°C for 24 hours. After this period the cells were loosened from the agar surface with a wire and washed off with approximately 10 ml of 0.15 M NaCl containing 0.3% formalin. The volume of bacterial suspension was generally 450-500 ml from 40 Roux bottles and 650-700 ml from 60 Roux bottles.

The bacterial cells were also grown in liquid media of Adams and Roe (1945) described for the cultivation of pneumococcus except Thioglycollate was omitted. The organisms were adapted to the media in broth tubes and then inoculated into 4 liter Erlenmeyer flasks containing 3 liters of Adams-Roe media.

Isolation of the Immunogenic and Toxic Complex

The Pasteurella multocida cells were washed off the surface of agar media with formalinized saline, stirred as a suspension for 1-3 days at 4°C and the cells removed by centrifugation at 13,200 x g for 30 min at 4°C. The packed cells were weighed wet and aliquots were dialyzed and lypholized for the estimation of dry weight. The culture supernatant was then filtered through a series of Millipore membrane to remove additional cells or cell fragments. The Millipore membranes were washed prior to use with boiling

water in a method described for removing the detergent contained in the membranes (Cahn, 1967). The fluid was filtered in succession through membranes having porosities of 1.2, 0.65 and 0.45 μ. On occasion the culture fluids were also filtered through membranes with porosities of 0.3 and 0.22 μ or through Seitz filter pads. The culture filtrate was then centrifuged in a Spinco No. 50 titanium rotor at 40,000 rpm (105,000 x g) for 2 hr at 4-10° C, using a Spinco Model L or Model L-2 preparative ultracentrifuge (Beckman Instruments, Inc.). The general procedure was to centrifuge a 120 ml portion of the culture filtrate, using 10 ml polycarbonate "Oak Ridge" type tubes, remove the top 9 ml of the supernatant with a syringe and cannula, resuspend the pellet in the bottom ml of solution, and allow it to stand overnight at 4°C. The following day the tubes containing the suspended material were refilled with culture filtrate and re-centrifuged. This procedure was repeated until all the culture filtrate had been centrifuged.

The total sediment from the culture fluid was then re-suspended in approximately 120 ml of formalinized saline and re-centrifuged three times at 40,000 rpm. Each time the pellet was allowed to re-suspend overnight at 4°C or in some instances gently re-suspended using a 5 or 10 cc syringe with a No. 18 cannula. After the third wash the pellets plus the bottom 1 ml of solution were re-suspended in 20 or 25 ml of either 0.15 M NaCl or distilled water containing

0.1% formalin. The resulting opalescent solution was designated the 40,000 rpm precipitate or 40-P immunogenic complex of Pasteurella multocida P-1059 Gray.

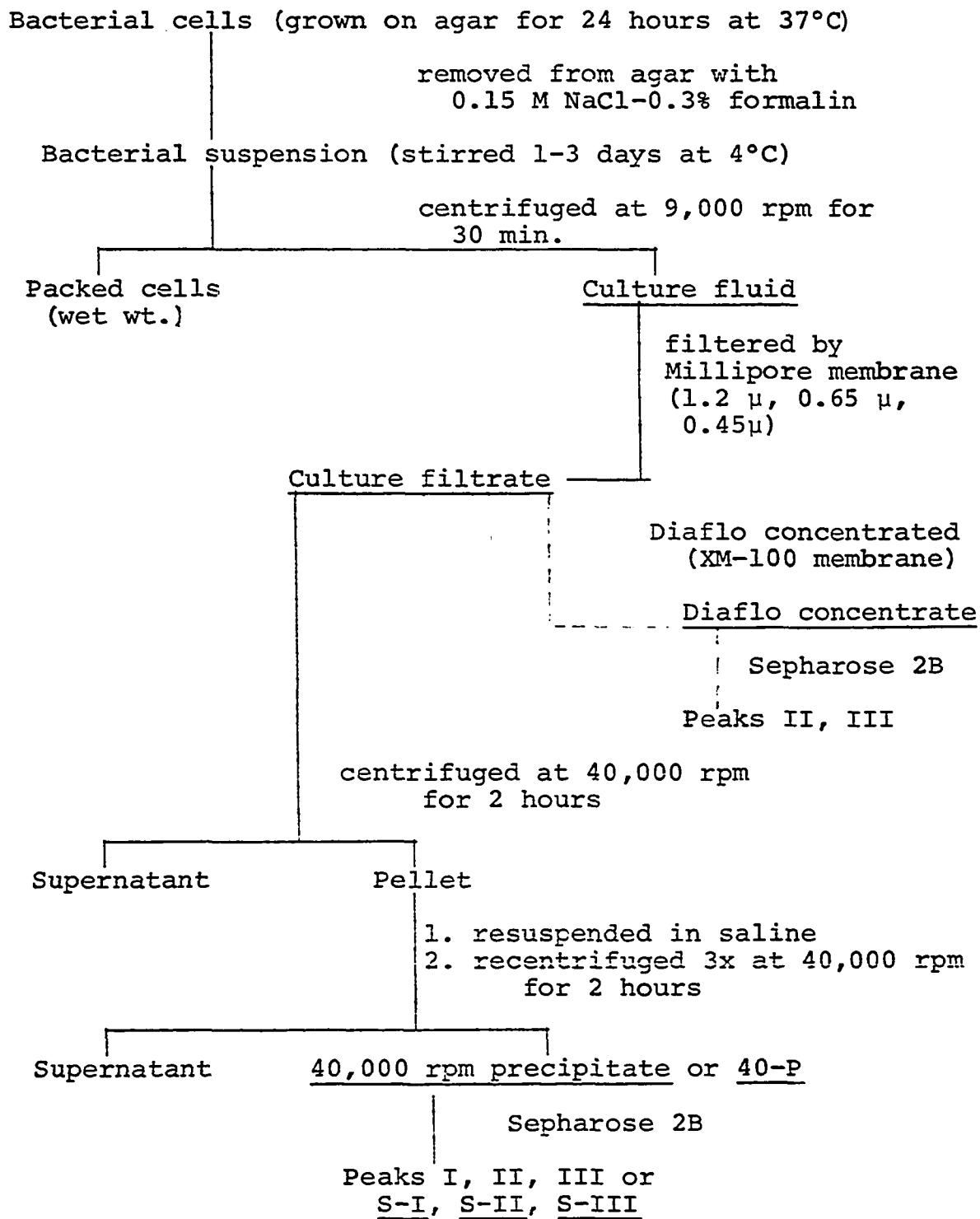
Special methods of preparation

The above general procedure was varied in several instances to study the effect in yield and on the chemical composition of 40,000 rpm precipitate. These methods are described in Results section.

Isolation of the complex from Adams-Roe culture fluid

The immunogenic complex was also isolated from the culture fluid obtained from the growth of P. multocida in a liquid media similar to one described by Adams and Roe (1945). Organisms were inoculated into three Erlenmyer flasks containing 3 liters of media each. Cultures were maintained at 37°C for 24 hr., after which the cells were removed using a Sharples centrifuge.

The culture fluid was then placed in washed cellulose casings size 1-7/8" inflated diameter and concentrated by placing the cellulose casings in a large pan and covering each bag with powdered polyvinylpyrrilidon. More polyvinylpyrilidon was periodically layered on the bags which were kept in a cold room at 4°C. After concentration of the fluid to a small volume (3 liters to 135 ml), the outside of the bags were washed with distilled water, the contents removed



Flow chart 1. Diagram of the isolation and purification of the P. multocida immunogenic complex

and filtered through a glass fiber filter, then through Millipore membranes and the complex isolated by centrifugation as described previously.

Chemical Methods

The total phosphorus content of the complex preparations was determined by p-semidine method of Dryer et al., 1957 and total nitrogen by the micro-Kjeldahl method outlined by Kabat and Mayer (1961, p. 480).

All colorimetric or ultraviolet absorption readings were made on a Zeiss PMQII spectrophotometer. Ultraviolet or visible absorption spectra were obtained through the use of a Beckman Model DK-2A recording spectrophotometer.

Methods of carbohydrate analysis

Total carbohydrate content of the preparations and fractions were determined by the phenol-sulfuric acid method of Dubois et al. (1956), or by modification of it, in which one-half amount of the reagents were used. In this procedure, to each sample in 1 ml saline, 25 µl of 80% phenol was added, the tubes were shaken and then 2.5 ml of concentrated sulfuric acid was added rapidly. After 30-60 minutes, the absorption of the solutions was measured in a Zeiss PMQII spectrophotometer at 490 nm. Glucose was used as standard.

Heptose was determined by the Dische cysteine-sulfuric method (1955) and also by Osborn's (1963) modification of this method using α-D-glucoheptose as a standard. The hexosamine content was determined by the procedure of Rondle and Morgan (1955) and the glucose and galactose content was measured enzymatically by Glucostat and Galactostat respectively.

Qualitative analyses of sugars in hydrolyzates were carried out using both paper and thin layer chromatography.

1. Paper chromatography - Separation of the sugars was carried out by descending paper chromatography using butanol-pyridine-water (6:4:3) as developing solvent. The sugars were visualized by an alkaline $AgNO_3$ dipping reagent (Smith, 1960).

2. Thin layer chromatography - The monosaccharides released by hydrolysis of the antigen with 2N sulfuric acid for 4 hr. were subjected to thin layer chromatography on glass fiber sheets impregnated with silica gel (ITLC-SG, a product of Gelman Inst. Co.).

Prior to use the glass fiber sheets were impregnated with 1% NaH_2PO_4 (w/v) and allowed to dry 1 to 3 days (Kraeger and Hamilton, 1969). The plates were developed in either $CHCl_3$-MeOH-pyridine-H_2O (130:40:1:5) or acetone-butanol-water (4:5:1). The latter solvent system is a modification of the system used by Bleiweis and Coleman (1969). The sheets were developed in an Eastman sandwich chamber developing apparatus. For the first solvent system three developments of 15 min each

were used and for the second solvent system two developments of 30 min each were used. The sugars were visualized by spraying with 0.3% p-anisidine-HCl in butanol and heating at 120°C for 10 min, a procedure reported to give a characteristic gray-green color for aldoheptoses (MacLennan and Davies, 1957).

Methods of protein analysis

The total protein content was estimated by the ultra-violet absorption method of Groves et al. (1968) and also by a modified Folin-Lowry method using bovine serum albumin as a standard (Bailey, 1967).

Amino acids were chromatographed from hydrolysates of 1 mg samples in 2 ml of 6N HCl, in a boiling water bath for 24 hrs. The HCl was removed by rotary evaporation. The developing solvent was butanol-acetic acid-water (60:15:25) prepared as a single phase (Smith, 1960). After development for 18 hours the papers were sprayed with either 0.3% ninhydrin in alcohol for detection of amino acids or the Elson-Morgan reagent (Smith, 1960) for the detection of amino sugars. This same procedure was used to chromatograph amino acids released by proteolysis with Pronase.

Method of Pronase digestion

The 40,000 rpm precipitates were subjected to proteolysis with the enzyme Pronase.

Incubation was carried out at 40°C for 6 hours in a total volume of 1 ml of phosphate buffer, pH 7.4, I = 0.05. Free amino acids were analyzed by paper chromatography. A similar preparation hydrolysed by 6N HCl for 24 hours was simultaneously chromatographed.

Methods of lipid analysis

Lipid was extracted from the 40-P complex with 2 volumes of $CHCl_3$-MeOH (2:1) three times. The extract and the water phase were evaporated to dryness under a stream of nitrogen. Both fractions were analyzed for ester content using the alkaline hydroxylamine method of Snyder and Stephens (1959). Tripalmitin was used as standard.

For thin layer chromatography of the lipids both precoated plates (ITLC-SG) and regular plates coated with silica gel G were used. The plates were developed in $CHCl_3$-MeOH-H_2O (65:25:4) and the spots visualized by spraying for phospholipid (Dittmer and Lester, 1964) or with 50% H_2SO_4. Phosphatidyl ethanolamine, phosphatidyl glycerol and phosphatidyl serine were used as standards and were obtained from sources described in Methods.

Stability studies of the immunogenicity of the 40,000 rpm precipitate

The immunogenic material isolated by repetitive centrifugation at 40,000 rpm was subjected to various treatments in order to determine their effects on the ability of the prepa-

ration to provide immunological protection to turkey poults challenged with virulent P. multocida. These treatments were:

1. Incubation in 0.1N NaOH (pH 13.0) for 4 hours at 40°C followed by neutralization with 0.1N formic acid

2. Incubation in 0.1N formic acid (pH 2.3) for 4 hours at 40°C followed by neutralization with 0.1 N NaOH

3. The complex in sterile saline (0.15M NaCl) was heated at 121°C for 1 hour in an autoclave

4. The complex (130 µg) was incubated at 40°C for 6 hours with 200 µg Pronase in a volume of 1.34 ml phosphate buffer pH 7.4 (I = 0.05).

Physical Methods

Fractionation and determination of the particle size by gel-filtration

Agarose gel filtrations were performed using Pharmacia columns K 25/45 and Sepharose 2B and 4B. All columns were prepared by pouring the entire volume of agarose at one time through the use of a connecting column reservoir (Pharmacia Fine Chemicals). All columns were slightly compacted with the use of a Pharmacia flow adaptor and packed with a flow rate greater than that used in experiments.

Samples were applied with the use of Luer-lok syringes through a three way valve located between the eluant reservoir and the column. Two or three ml samples were applied and flow

rates were maintained between 10 and 11 ml/hour by keeping a constant pressure head of 9 cm. In most cases 5 ml fractions were collected by drop counting using a LKB fraction collector. Generally 250 ml was collected over a 22-23 hours period using 0.15M NaCl containing 3% ethyl alcohol as the eluant. Both ascending and descending chromatography were used.

The fifty fractions were each examined for ultraviolet absorption in a Zeiss PMQII spectrophotometer at four different wavelengths: 280, 260, 233 and 224 nm (nanometers) in 1 cm cells. Total nitrogen, carbohydrate and protein content of the fractions were determined by methods described in the Chemical Methods section.

Determination of column parameters

The elution volume (V_e) was determined from fraction which had the maximum absorbance at 224 nm for P. multocida preparations, 260 nm for ribosomes and 280 nm for Blue Dextran. The void volume (V_o) was determined from the elution volume of the largest component of Blue Dextran. The value for internal volume (V_i) or the total imbibed volume of the column was obtained from the difference between the elution volume of formaldehyde and the void volume. The presence of formaldehyde was detected with a Schiff's reagent (Block et al., 1958). The total bed volume (V_t) was calculated from the dimensions of the column.

Determination of K_d The distribution coefficient K_d was determined using the basic equation of Gelotte (1960) which is:

$$V_e = V_o + K_d V_i \quad .$$

In this equation, the effluent peak volume (V_e) is related to the void volume (V_o) and the internal volume (V_i), i.e. the volume of solvent internal to the gel phase. This equation can be rearranged to:

$$K_d = \frac{V_e - V_o}{V_i} \quad .$$

From this equation it can be seen that for solutes totally excluded from the internal volume $K_d = 0$ and $K_d = 1$ for those in which the solvent both within the pores and in the void volume are equally accessible.

Determination of K_{av} The method of Laurent and Killander (1964) was used to calculate another distribution coefficient (K_{av}). This coefficient is defined as the fraction of the total gel phase available to a solute molecule and depends on the consideration of the entire gel phase as the stationary phase. The K_{av} can be calculated from the following equation:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \quad .$$

Determination of Stokes radius The effective particle size or Stokes radius a can be calculated from the value of the gel filtration distribution coefficient K_d using the Ackers' (1964) relationship:

$$K_d = \frac{V_e - V_o}{V_i} = \left(1 - \frac{a}{r}\right)^2 \left[1 - 2.104 \frac{a}{r} + 2.09 \left(\frac{a}{r}\right)^3 - 0.95 \left(\frac{a}{r}\right)^5\right]$$

This equation is unique in that only one constant r is used and this is the effective pore radius of the gel. Use of this equation is made more valuable by the publication by Ackers of parameters for column calculations which relate the Stokes radius and effective pore radius a/r to the distribution coefficient K_d. In this manner the determination or assumption of a given value of r allows one to directly calculate the Stokes radius of a macromolecule from the distribution coefficient.

Estimation of sedimentation coefficient The sedimentation coefficients were determined through the cooperation with Dr. Marshall Phillips (National Animal Disease Laboratory, Ames, Iowa). The Spinco Model E Analytical Ultracentrifuge (Beckman Instruments, Inc.) was used with Schlieren optics. The experiments were performed at ambient temperature and 29,500 rpm on samples previously dialyzed against formalinized saline (pH 5.5 - 6.5). Samples containing 2.4 to 7.2 mg/ml of the 40,000 rpm precipitate as well as samples further purified by Sepharose 2B gel filtration (peak II) which

contained 9 mg/ml were used. The sedimentation coefficients were calculated using the method of Chervenka (1969) and the values obtained were adjusted to correspond to sedimentation rates in water at 20°C.

Estimation of molecular weight The molecular or particle weight was estimated from the combination of the gel-filtration value for the Stokes radius and the sedimentation rate. This method is described by Ackers and Steere (1967) and involves the estimation of the diffusion coefficient by the Einstein relationship:

$$D_{20,w} = \frac{RT}{N6\pi\eta a}$$

where D is the diffusion coefficient at 20°C in water, R the gas content, T the absolute temperature, N Avogadro's number, η the viscosity of water at 20°C and a the Stokes radius.

The determined diffusion coefficient is then used with the sedimentation coefficient ($S_{20,w}$) in the Svedberg equation to estimate the anhydrous molecular or particle weight (M):

$$M = \frac{RTS_{20,w}}{D_{20,w}(1-\bar{v}\rho)} \quad .$$

In this equation $\bar{v}$ is the partial specific volume and ρ the density of water at 20°C and other terms as described above.

Potassium bromide density gradient centrifugations

Potassium bromide (KBr) gradients were prepared by layering aqueous solutions of KBr in the cellulose nitrate (5 ml) tubes with a pipet. After 30 min the samples and various density markers were added. Enough crystalline KBr was added to each sample so that it contained a total KBr concentration approximately 4% w/w less than the top of the prepared gradient. The tubes were centrifuged[1] at 40,000 or 60,000 rpm and 25°C. after 18 - 24 hour time intervals the centrifuge was stopped and the positions of the density markers in relation to light scattering zones was noted. A narrow beam of light shown from directly above the tube aided in the detection of the zones. When the relative positions of the density markers and the light scattering zones failed to change, the tube contents were fractionated by removing 0.25-0.30 ml fractions either by puncturing the bottom of the tube or by withdrawal of samples from the meniscus with a pipet. The refractive index of each fraction was determined with a Zeiss refractometer and the density calculated through the use of an equation (Ifft and Vinograd, 1966) which relates the density of KBr (ρ) to its refractive index (η).

$$\rho^{25°} = a(\eta^{25°}) - b \quad \text{where} \quad a = 6.4786$$
$$b = 7.6431 .$$

[1] In Beckman L-2 or L-4 ultracentrifuge with SW-65 rotor.

Three different linear gradients were formed by varying the volumes and concentrations of KBr solutions as described below.

(a) 1.2 ml of each of four aqeous solutions of KBr (w/w) 24%, 28%, 32% and 36%. Density range 1.17-1.34.

(b) 1.6 ml of three KBr solutions 28, 32, and 36%. Density range 1.22 - 1.34.

(c) 2.4 ml of 32% and 36% KBr solutions. Density range 1.26 - 1.34.

The 17-20 fractions were examined for their serological reactivity in Ouchterlony gel diffusion plates. The best results were obtained when the hyperimmune rabbit antiserum contained an approximately equal concentration of KBr.

Each fraction was also examined for its ultraviolet absorption at 280 nm. The fractions were diluted either three or five fold with distilled water and the absorbance determined using micro-cuvettes. The blank was a 36% w/w KBr solution diluted an equal amount.

Sucrose density gradients centrifugations

Sucrose density gradients centrifugations were performed in much the same manner as the KBr gradients. Equal volumes of approximately 1.2 ml of 45, 50, 55 and 60% w/w sucrose solutions containing 0.15M NaCl were layered, the tubes allowed to stand overnight at room temperature and the samples in 40% sucrose and density markers were applied the next day.

Centrifugation was performed at 60,000 rpm and 22°C for time periods of 24-72 hours (using SW-65 rotor in Beckman L-2 ultracentrifuge).

Electrophoretic methods

The immunogenic and toxic preparations isolated from P. multocida P-1059 Gray were subjected to various electrophoresis techniques in order to determine the relative homogeniety of the preparations as well as their composition, isoelectric point, and mobility in different media. The electrophoretic techniques used include acrylamide-agarose gel electrophoresis, cellulose acetate electrophoresis and isoelectric focusing. Immunoelectrophoresis experiments were also performed and the methods used in this procedure are described in the section on serological methods.

Acrylamide-agarose gel electrophoresis A new method of electrophoresis in composite acrylamide-agarose gels was adapted to the analysis of the immunogenic complex isolated from P. multocida. This method was similar to that described by Peacock and Dingman (1968) for the separation of ribonucleic acids and also to one described for the separation of proteins (Uriel, 1969). The optimum concentrations of acrylamide and agarose which allowed a significant migration of the complex antigen were 2.5% w/v acrylamide and 0.8% w/v agarose. A 1.06% w/v agarose solution in 0.057 M Tris-HCl buffer pH 8.9 was prepared by melting the agarose at 121°C for 5 min and then cooled to 50°C. A 10% w/v acrylamide solution

was prepared using the same buffer.

The composite gel was formed as follows: the 10% acylamide solution was warmed to 50°C, then one part of the acylamide solution was added to three parts of the 1.06% agarose solution, enough ammonium persulfate catalyst was added to make a concentration of 0.07% w/v, the mixture was quickly stirred with a Vortex mixer and then pipetted into 4.5 x 62 mm glass columns with a Pasteur pipet. The columns previously had been slightly constricted at one end by heating with a gas-air torch in order to prevent the gels from sliding out during electrophoresis. After the gels were firm (about 2 hours later at room temperature) they were placed in the Canalco Disc Gel Apparatus (Canal Industrial Corporation). Samples of the 40,000 rpm precipitate and a preparation purified further by Sepharose gel filtration (i.e. peak II) as well as the proteins ferritin and bovine serum albumin were applied to the surface of the gels. The samples were layered beneath the buffer solution covering the gels through the use of dense sucrose solutions. Approximately 0.2 mg samples were applied in volumes of 0.15 to 0.30 ml. The anode and cathode buffers were 0.025 M Tris-glycine pH 8.3. Electrophoresis was carried out for 1-1/2 hours at 2.5 ma per tube. The gels were stained with one of the following: Bromphenol Blue for protein, Sudan Black B (a saturated solution in 60% ethanol diluted two fold prior to use with 60% ethanol) for lipid

(Noble, 1968) or a periodic acid-Schiff's reagent for glycoproteins (Zacharius et al., 1969).

Cellulose acetate electrophoresis Electrophoresis of the toxic and immunogenic preparations was performed using two types of cellulose acetate strips. One type was a product of Colab, Inc. (Cellogel, a gelatinized cellulose acetate) and the other was Sepraphore, a cellulose acetate strip produced by Gelman Instruments Co. Eight microliters of the preparations were applied to each of six strips of Sephaphore (2-1/2 x 17 cm) or Cellogel (2-1/2 x 14 cm) which had been previously soaked in 0.05 M sodium borate pH 9.0 for approximately 30 minutes. The samples were allowed to soak into the strips for five minutes and then electrophoresis was performed for one hour using 140 V and an initial current of 20 ma. A "Shandon" electrophoresis apparatus (Colab Laboratories) was used. At the conclusion of the electrophoretic run two strips were placed in each of three solutions containing one of the following reagents: Amido Black 10 B for staining of proteins (procedure described by Colab Lab. Inc.), Fat Red 7 B for the detection of lipids (Straus and Wurm, 1958), and a periodic acid - Schiffs' reagent for the staining of glycoproteins (Zacharius et al., 1969).

Isoelectric focusing The LKB model 8100 electrofocusing equipment (LKB - Produckter AB) was used to study the homogeneity and the isoelectric point of the P. multocida

P-1059 toxic and immunogenic preparations. Electrofocusing was carried out as described by the LKB Instruction Manual. Experiments were performed using both pH 3 to 5 and pH 3 to 10 ampholine solutions. The time of electrofocus was 20 hours with the pH 3-10 gradient and 72 hours with the pH 3-5 gradient. All experiments were performed using the anode at the bottom of the Model 8102 column and the cathode at the top. The voltages used for pH 3-10 and pH 3-5 experiments were 300 and 450 volts respectively.

The sucrose gradients were made manually as described by LKB with the sample added to the middle of the gradient. The column was maintained at 35°F through circulation from a refrigerated bath, and the 3 ml fractions were collected at the conclusion of the run using an LKB fraction collector. The electrofocus runs were all concluded after the current fell to a minimum and persisted there for several hours.

Biological Methods

Preparation of rabbit antisera

Antisera to the 40-P preparations was prepared by injection of white New Zealand rabbits by three general procedures. Procedure (1) the rabbits were injected with 0.1 ml or 0.2 ml solutions of the immunogenic preparation intravenously in the marginal ear vein. Procedure (2) the rabbits were injected intradermally with 0.2 ml of preparation in each

of five sites on the shaved backs. These antigen solutions were injected either with adjuvant or without. The emulsified adjuvant inoculum was prepared by emulsification of a volume of aqueous antigen preparation with an equal volume of 3% Arlacel A in Bayol F. Procedure (3) the rabbits were injected intramuscularly in the thigh of the hind limb. The rabbits were bled either by heart puncture or from the ear vein. Generally, injections and bleedings were at 2-4 week intervals. The exact schedule for 2 rabbits used most frequently can be seen in the Results section.

Immunity studies with turkey poults and mice

One week old turkey poults were given 0.1 ml intravenous injections in the wing or intramuscular injections in the thigh of the preparations being evaluated for their immunogenicity. They were challenged from 2-1/2 to 3 weeks later with approximately 10^3 virulent P. multocida P-1059 F1 organisms. The actual number of organisms injected in the challenge was estimated from colony plate counts and is reported in the Results section. The turkey poults were observed for approximately two weeks following challenge.

The 16-18 gm female Swiss Webster mice used in immunity studies were injected intraperitoneally with 0.1 or 0.2 ml of the preparations being tested for immunogenicity. The mice were challenged with virulent P. multocida P-1059 F1

organisms (approximately 10^3 colony forming units) at either five or seven weeks following the initial injection of preparations.

Passive immunity studies with rabbit antisera

The ability of rabbit antisera to the immunogenic 40-P preparations to passively protect mice against infection with P. multocida P-1059 F1 was determined. The 0.2 ml volumes of rabbit antisera were injected into the peritoneal cavity of 16-18 gm female mice. Twenty-four hours later the mice were inoculated intraperitoneally with approximately 10^3 virulent P. multocida P-1059 organisms. The mice were observed for at least two weeks following challenge.

Lethal toxicity studies in chick embryos and mice

The amount of preparation which was lethal for 50% of the injected animals (LD_{50}) was determined with chick embryos and mice. The following two procedures were used:

(1) Nine to ten day old chick embryos were inoculated in a procedure similar to the method described for the virus incubation in chick embryos (Horsfall and Tamm, p. 1205, 1965). An artificial air sac was created on the side off the embryo by drilling two holes. The first hole was drilled with a small hand vibrator through the end of the egg into the natural air sac and then a second hole was drilled on the side of the egg cautiously so as to not pierce the membrane just

inside the shell. A gentle suction with a small bulb was applied to the first hole to create the artificial air sac. The air sacs were formed in 9 or 10 day old embryos and the following day 0.1 or 0.2 ml of the preparations were inoculated with a one cc syringe and 27 gauge needle onto the chorioallantoic membrane through the lateral hole. Both holes were then sealed with plastic cement. Control embryos were injected with the diluent (0.15 M NaCl containing 0.1% w/v formalin). The embryos were incubated at 37°C and candled at least twice a day for one week following inoculation. Approximately eight dozen embryos were used in each experiment with ten or twelve embryos per dose of inoculum.

The number of deaths occurring within 48 hours of inoculation was recorded and the number of deaths per number of inoculated embryos at each dose was submitted to probit analysis for the determination of the 50% lethal dose (LD_{50}) and 95% confidence limits.

(2) Determination of the LD_{50} in 80 mice was carried out as follows: Randomized 16-18 gm mice were divided into 5 groups of 16 and each mouse was injected (i.p.) with 0.2 ml of a dilution of the toxic preparation. The mice were observed at various time intervals for at least two weeks after the injection of toxin.

The number of deaths per number of mice inoculated with each dose was recorded and the data submitted to probit

analysis through the use of a computer for determination of the LD_{50} and 95% confidence limits.

Serological Methods

Immunoelectrophoresis

The micro-scale method of immunoelectrophoresis described by Grabar and Williams (1953) was used to study the immunogenic complex at various stages of purification. Three ml of 0.6% "Seakem" agarose solution in a Tris-citrate (0.02 M Tris-0.02 M sodium citrate) buffer adjusted to pH 8.6 with HCl was pipetted onto a microscope slide. Samples were applied to wells cut in the cooled agarose gel and electrophoresis was carried out at 20 v and 80-100 ma for 45-60 minutes. At the completion of electrophoresis, a center trough was cut, removed, and antiserum applied. Immunodiffusion was allowed to proceed for 1 to 3 days before photographing the resulting precipitin lines.

Immunodiffusion

Agarose gel diffusion plates were prepared by suspending 4 g of "Seakem" agarose in 500 ml of Tris-citrate buffer (0.02 M Tris-0.02 M sodium citrate) pH 7.2. The agarose was melted in an autoclave at 121°C for 5 minutes, 5 ml of 6% phenol-1% thimersol solution was added, and then 25 ml was pipetted into 90 mm petrie dishes. Holes were cut in the agarose gel plates with Feinberg gel cutters or through the

use of plexiglas templates and a No. 1 cork borer. Two plexiglas templates were used. The distances between the edges of the 5 mm holes was 1 mm in one template and 1-1/2 mm in the other. In all instances the holes were completely filled.

RESULTS

Reports of previous investigations in this laboratory (Rebers et al., 1965 and Heddleston et al., 1966) have described the isolation of a toxic and immunogenic substance by ultracentrifugation of saline extracts of two avian strains of P. multocida. These preparations were serologically complex when examined in Ouchterlony type gel diffusion plates. Although in some respects they resembled typical endotoxins, the use of established methods of endotoxin isolation such as the phenol extraction method (Westphal et al., 1952) resulted in preparations which failed to immunize fowl against infection with P. multocida. However, the phenol extracts as described later did give one or more precipitin lines in Ouchterlony plates when examined with rabbit antisera to the ultracentrifuged sediment. The phenol extracted material did not appear to be serologically related to any of the major antigenic components of the sediment obtained by ultracentrifugation. The ultracentrifugation procedure used as a primary step in isolation is described in the methods section and shown on the flow sheet on page 30.

The usual methods of isolating endotoxins or lipopolysaccharides from bacteria involve rather drastic conditions of extraction which in all probability greatly alter the natural state of the toxic and antigenic components of gram-negative cells. In contrast, the results described here are from the

use of experimental procedures designed to retain as much as possible the natural state of the toxic, antigenic, and immunogenic substances.

Isolation Procedures

Since the primary objective of this investigation was to isolate an immunogenic fraction from P. multocida, the preliminary experiments were designed to isolate the substance using as gentle an isolation process as possible in order to maintain the natural state of any immunogenic substance. The ultracentrifugation procedures of centrifuging the culture filtrates at 40,000 rpm (105,000 x g) for 2 hours (at 4-10°C) resulted in a sediment that was immunogenic for turkey poults. It was found that 3 repeated centrifugations of the resuspended sediments using the same conditions removed small amounts of material (presumably proteins) that absorbed at 280 nm in the ultraviolet. The yield of this particulate immunogen which is sedimented by ultracentrifugation would be expected to be a function of both the centrifugal field and time. Two sets of conditions were found which gave workable yields of immunogenic sediment. The first is the one described above. Recentrifugation of the 40,000 rpm supernatants at 50,000 rpm (165,000 x g) for 2 hours resulted in little additional sediment. The second effective procedure was to centrifuge the entire volume (500-700 ml) of culture filtrate overnight (16 hours) at 30,000 rpm (78,000 x g) at 4°C. This sediment

was then re-centrifuged three times at 40,000 rpm for 2 hours to remove some of the lower molecular weight components.

The resulting immunogenic sediments were compared for total yield and by chemical analysis (nitrogen, phosphorus, and carbohydrate) and found to be very similar. These sediments were designated as 40,000 precipitates (40-P) and formed the starting material for subsequent fractionations.

Ultrafiltration of the culture filtrates through Diaflo membranes (X-M 100) was found to be effective in reducing the volume of the culture filtrates to approximately 120 ml. These membranes are reported to retain and concentrate macromolecules of 100,000 molecular weight or larger. The retained fluid was then ultracentrifugated at 40,000 rpm for 2 hours and washed three times in the same manner. The materials isolated in this way did not appear to differ antigenically or chemically from the usual 40-P material.

The antigenic complexity of the various supernatants, sediments, and washings was determined by Ouchterlony type gel diffusion plates using rabbit antisera prepared against either the whole saline extract of the organisms or antisera to the 40,000 precipitate. The washed 40-P preparations were found to give one or two slow diffusing antigens depending on concentration while the washings of those sediments showed numerous precipitin lines which formed close to the antisera well indicating their lower molecular weight.

The three additional ultracentrifugations of the initial sediments appeared in each case to reduce their antigenic complexity. If the initial sediments were washed only once or twice their nitrogen contents were higher and their ultraviolet spectra were indicative of a higher protein content. If the initial sediments were washed by repeated ultracentrifugations at 105,000 x g for as many as five to six times, there was a dramatic decrease in yield of material and immunodiffusion plates indicated the antigens were not being resedimented.

The yield of the 40-P preparations was determined by dialysis against distilled water and lypholization of aliquots. The average yield of 40-P material was 0.206% (w/w) of the packed wet weight of cells. Eight different preparations from different cultures were averaged with the individual yields varying from 0.13% to 0.37%. There were no apparent differences in chemical or serological analysis which could be correlated with individual variation of the yield.

The general method of extracting the cells grown on agar medium with isotonic saline is described in the methods section. Although the general procedure was to wash the loosened cells off the agar surface and allow them to stir for one to three days before removing the cells, it was found that 80% of the total 40-P material could be isolated if the cells were stirred for only one hour. This fact suggests that the recovered immunogenic and toxic material is actually excreted

from or very loosely bound to the surface of the bacterial cells.

Later in the course of this investigation P. multocida type P-1059 Gray organisms were grown on a liquid medium. In this case the immunogenic and toxic 40-P material could be isolated from the concentrated culture filtrate.

Biological and Serological Studies of the 40,000 rpm Preparations

Throughout the investigation of the material sedimented from the culture filtrates of P. multocida P-1059, experiments were carried out to study the biological properties of the isolated material. Of primary interest was the ability of these preparations to protect animals against lethal infections of Pasteurella multocida. In addition the preparations were found to be toxic and to produce precipitin type antibodies when injected into rabbits. The rabbit antisera to the 40-P preparations were capable of providing passive immunity in mice.

Immunogenicity of the preparations

Since the organism P. multocida under study causes acute infections of birds and the strain P-1059 was originally a turkey isolate, it was felt best to evaluate the protective or immunogenic properties of the isolated materials in turkeys whenever possible. The results of an experiment where a 40-P

preparation was used to actively immunize two week old turkey poults are summarized in Table 1. A single injection of ten micrograms of the crude 40-P material was found to immunize 70% of the turkey poults when challenged two weeks later with approximately 10^3 virulent organisms. One half (5) of each group was injected intravenously in a wing and the other half intramuscularly in a thigh with the 40-P preparations. There was no apparent difference in the survival rate of the turkeys which had been injected by these two routes.

Table 1. The effect of different doses of a 40-P preparation on the protection of turkeys against challenge inoculation with P. multocida P-1059 Fluorescent[a]

Doses of 40-P[b] (µg)	No. dead on day 1	2	5	7	No. survivors / No. challenged	% Protection
10	0	1	1	1	7/10	70
1	3	1	3	0	3/10	30
0.1	8	0	1	1	0/10	0
Control	10	0	1	0	0/11	0

[a]2 week old Beltsville Small White Turkey poults, one half (5) of each group injected intramuscularly (thigh) and rest intravenously (wing vein). Two weeks later their immunity was challenged with intramuscular injections of 885 P. multocida P-1059 F (determined by colony plate count). Poults were observed for 2 weeks following challenge.

[b]40-P preparation lot 80 contained 4.26% Nitrogen, 19.4% carbohydrate, and 2.02% Phosphorus.

Stability of the immunogenicity Experiments were also carried out to test the stability of the immunogenic properties of the 40-P preparations. In this study the 40-P material was subjected to proteolytic digestion (Pronase), dilute acid (0.1 N formic acid), dilute base (0.1 N NaOH) and heat (60 minutes at 121°C in an autoclave). The results of this experiment are summarized in Table 2. The immunogenicity of the preparation did not appear to be affected by a Pronase solution which was active when assayed by the hydrolysis of casein. The immunogenicity appeared to be slightly decreased by formic acid (from 70 to 50%). There was a slight precipitate when the 40-P preparation was added to the formic acid which was re-suspended and inoculated in the suspended form. As shown in the table the incubation with alkali decreased (from 70 to 10%) the ability of the 40-P material to protect the inoculated turkey poults. The normally opalescent solution of 40-P material immediately became clear after the addition of the sodium hydroxide. However, there was no precipitation. The subjection of the 40-P material to heat did not have a visual effect on the solution but the immunogenicity of the preparation decreased from 70% to a 10% protective level.

The above treated and untreated solutions were injected intramuscularly in the thigh of the 2-1/2 week-old turkey poults. Each poult received 10 micrograms of the immunogen

Table 2. Studies on the stability of immunogenicity of 40-P preparations from P. multocida P-1059 Gray as evaluated by challenge of immunized turkey poults with virulent P. multocida P-1059 Fluorescent[a]

Treatment of 40-P immunogen	No. dead on day 1	2	5	7	No. survivors / No. challenged	% Protection
Untreated 40-P	1	0	2	0	7/10	70
Pronase digestion[b]	0	1	1	1	7/10	70
Incubation in 0.1 N formic acid[c]	1	3	1	0	5/10	50
Incubation in 0.1 N NaOH[c]	5	4	0	0	1/10	10
Heating in autoclave[d]	6	2	1	0	1/10	10
Non-vaccinated controls	4	6	1	0	0/11	0

[a] 2-1/2 week old turkey poults were injected intramuscularly with 10 µg of untreated or treated lot #80 immunogen in 0.1 ml volumes. Challenged 2 weeks later by intramuscular inoculation with 885 P. multocida P-1059 F. Observed for 2 weeks following challenge.

[b] 134 µg of immunogen, incubated 6 hrs, 40°C with 200 µg Pronase in PO_4 buffer, pH 7.4, I = 0.05.

[c] 134 µg, incubated 4 hours, 40°C. Then neutralized before injection.

[d] 134 µg in 1.34 ml of sterile saline placed in autoclave 121°C, 60 minutes.

in a volume of 0.1 ml. Prior to injection the alkali treated sample was neutralized with 0.1 N NaOH. The untreated and heated samples were injected as isotonic saline solutions and the pronase treated sample was in phosphate buffer.

Immunogenicity of the 40-P preparations was also studied in mice when turkey poults were not available. However, the results of these experiments are described in a following section of this dissertation which compares the immunogenicity of the 40-P preparations and fractions of the 40-P preparations obtained by agarose gel filtration.

Production of rabbit antisera

The 40-P preparations were found to produce precipitating antibodies when injected either intravenously or intradermally in the white New Zealand rabbit. Intravenous injections of saline solutions were performed in the marginal ear vein while intradermal injections of emulsified (3% Arlacel A in Bayol F) preparations were made in several sites on the shaved backs of the rabbits. Precipitating antibodies were generally detected two weeks later when the serum collected by bleeding from the ear vein was examined in Ouchterlony type immuno-diffusion plates.

The general schedule of immunization was to bleed the rabbits two weeks after the initial injection, re-inject the rabbits, and then bleed them from 2 to 4 weeks after subsequent injections. The dose of the initial injection was generally

less than 60 micrograms as higher doses frequently resulted in toxicity symptoms including paralysis of the hind limbs. After several injections of the lower dose the amount of 40-P injected could be increased several fold without the appearance of toxicity symptoms. The antisera which gave the strongest Ouchterlony precipitin lines, i.e., those in which the lines appeared early and more intense, were generally prepared from blood collected two weeks after an increase in the antigen dose.

The 40-P preparations gave 2 or 3 precipitin lines in the Ouchterlony immunodiffusion plates with antisera prepared from various bleeding and various rabbits. If the concentration of the 40-P preparation was less than 2 mg/ml, only one precipitin line was observed even when the most sensitive immunodiffusion pattern was used. A single precipitin line was observed using 40-P solutions as dilute as 0.1 mg/ml.

The schedule of bleedings and injections of the 40-P preparations in two rabbits whose antisera were used most frequently is summarized in Table 3.

Those antisera marked by an asterisk are those which gave the strongest precipitin lines when examined in Ouchterlony plates with the homologous 40-P preparation.

An example of the gel precipitin reactions is shown in Figure 1a. The 40-P preparations gave two precipitin lines when examined with almost all antisera prepared against the

Table 3. The production of rabbit antisera to the P. multocida P-1059 40-P preparations[a]

Amount of 40-P injected	Date of injection	Date of bleeding	Amount of 40-P injected	Date of injection	Date of bleeding
Rabbit #71			Rabbit #92		
90 µg	2-16-65	3-11-65	45 µg	9-28-65	10-25-65
--	--	3-23-65	"	10-28-65	11-5-65
180 µg	3-26-65	4-8-65	"	11-9-65	11-19-65
"	4-19-65	5-6-65	"	11-23-65	--
"	5-19-65	6-23-65	"	12-9-65	12-20-65
"	6-28-65	7-7-65	"	12-21-65	1-7-65
"	7-9-65	7-22-65	"	1-12-66	1-21-66
"	7-23-65	8-3-65	110 µg	1-26-66	2-4-66[b]
"	8-6-65	8-20-65	"	2-11-66	2-18-66[b]
450 µg	8-24-65	9-8-65[b]	"	2-25-66	3-4-66
"	9-14-65	9-23-65[b]	"	3-11-66	3-18-66
			"	3-25-66	4-24-66
			"	7-26-66	6-3-66
				--	6-29-66

[a]Both rabbits injected intravenously in marginal ear vein; rabbits were bled by heart puncture or from the ear vein.

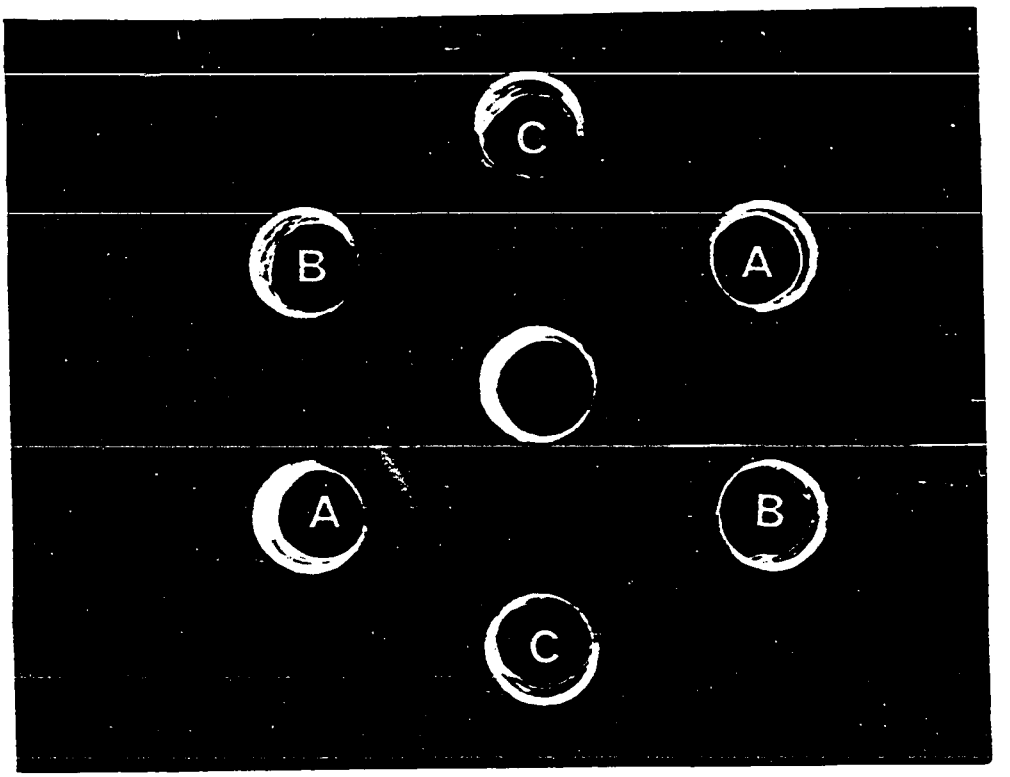
[b]These antisera gave the most intense precipitin lines when reacted with 40-P preparations in Ouchterlony plates.

Plate 1a. Immunodiffusion of antigens extracted from *P. multocida* P-1059 Gray

Wells labelled A contained a 40-P immunogenic preparation; B, a Sepharose 2B fraction (peak S-II); C, the aqueous layer of a hot phenol extract. The center well contains a hyper-immune rabbit antiserum (#92, 2-8-66) prepared from blood of rabbit injected 8 times over a 4 month period.

Plate 1b. Immunoelectrophoresis of a 40-P immunogenic preparation

Small wells contain the 40-P antigen and center trough the anti-40-P rabbit antiserum. Electrophoresis for 45 minutes at 20 volts, 80-100 ma, in 0.02 M Tris-Citrate buffer pH 8.6.


C
B
A
A
B
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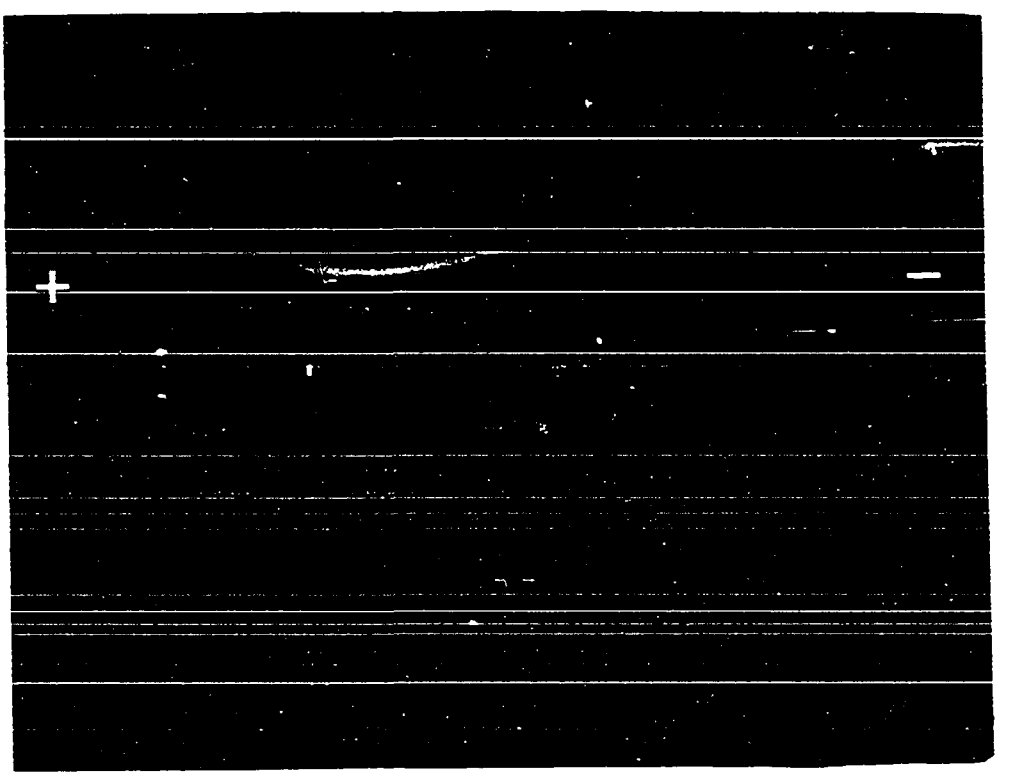

+
−

40-P preparations and at least 2 precipitin lines when antisera to the whole P. multocida P-1059 cells was used (see well labelled A). Some 40-P preparations gave a faint precipitin line closer to the antiserum well than the prominent lines.

Plate 1a also includes a comparison of phenol extracts of the Pasteurella multocida P-1059 Gray cells with the 40-P preparations. The phenol-extracted material (in wells labelled C) although apparently related serologically to the crude 40-P preparations as it reacts with the 40-P antisera, it does not appear to be identical to either prominent precipitin component of the 40-P preparations (in wells labelled A). The nature of the phenol-extracted antigen or antigens is not known but is currently being studied in a separate investigation in this laboratory. Immunity studies revealed that injection with the phenol-extracted materials failed to protect against infection with P. multocida.

The main gel filtration fraction S-II of the 40-P preparation (in wells labelled B) appears to contain the same two precipitin antigens found in the 40-P preparations as shown in Plate 1a. The results of other serological studies of the gel filtration fractions are described in a following section of this dissertation.

The results of an immunoelectrophoresis experiment are shown in Plate 1b. The 40-P preparations from various lots of cells consistently showed two precipitin lines in the positions

shown in the plate. The initial ultracentrifuge sediment (105,000 x g, 2 hrs.) of the culture filtrates usually contained numerous precipitin antigens when examined with homologous antiserum. However, after the washing by repeated centrifugation at 105,000 x g for 2 hrs. the number of observed precipitin components decreased to two. These two precipitin antigens appeared to have nearly the same mobility under various electrophoresis conditions as the center of the precipitin arcs occurred in approximately the same position in relation to the current flow.

Passive immunity studies with rabbit antisera

Passive immunity studies were carried out using rabbit antisera to the 40-P preparations, and 0.2 ml of this antisera was injected into the peritoneal cavity of 16-18 gm Swiss Webster mice. Twenty four hours later the mice were challenged with an intraperitoneal injection of approximately 10^3 virulent P. multocida P-1059. The results of one of these experiments is shown in Table 4. Rabbit antisera produced by intradermal injections of the antigen with adjuvant provided the best protection to the mice. The number of rabbits injected by the different routes was not large enough however, to definitely determine the best route of injection for producing protective antibody.

When the antisera used in the passive immunity studies were tested for their precipitin dilution titer in Ouchterlony

Table 4. Passive immunity studies of rabbit antisera to the 40-P preparations as evaluated by the protection of mice[a]

Preparation of rabbit antisera[b]		Protection of mice					
Rabbit no.	No. and method of injections	No. dead on day 1	2	4	7	No. survivors / No. inoculated	% Protection
108	3-intradermal with adjuvant	0	0	1	0	4/5	80
109	3-intradermal with adjuvant	0	3	1	0	1/5	20
110	3-intradermal with adjuvant	0	0	1	1	3/5	60
114	3-intramuscular	0	1	3	0	1/5	20
116	3-intradermal	0	1	3	0	1/5	20
117	3-intradermal	2	0	1	2	0/5	0
92	5-intravenous	0	0	3	2	0/5	0
120	Normal rabbit sera	5	-	-	-	0/5	0
---	No sera	5	-	-	-	0/5	0

[a]Each 16-18 gm mouse was injected (intraperitoneally) with 0.2 ml of rabbit antisera, 24 hrs later the mice were injected (intraperitoneally) with approximately 10^3 virulent P. multocida P-1059. Mice were observed for two weeks following challenge.

[b]Rabbits numbered 108, 109, 110, 114, 116, and 117 received 3 equal dose injections of 56 µg (lot 80) over a 16 week period; rabbit 92 had 5 intravenously injections of 45 µg (lot 32) over a 16 week period.

diffusion plates, there did not seem to be a direct correlation of the ability of the antiserum to passively protect mice and the concentration of precipitating antibody.

Lethal toxicity of the 40-P preparations

Since the 40-P preparations contained some of the components of endotoxins and toxic symptoms were evident when rabbits were injected for the production of precipitin antibodies, toxicity experiments were also performed. The results of a toxicity experiment using mice are shown in Table 5. Five groups of randomized mice were injected intraperitoneally with different amounts of the 40-P preparations. The 50% lethal dose (LD_{50}) was found to be 195 µg with upper and lower 95% confidence limits of 148 µg and 264 µg respectively. These values were determined by the probit method.

The toxicity of a similar 40-P preparation for chick embryos was investigated in an experiment whose results are shown in Table 6. Formalinized saline solutions containing various concentrations of the 40-P preparation were inoculated onto the chorio-allantoic membranes of the 10-day-old chick embryos. The embryonated eggs were candled several times daily and deaths recorded. Embryos where there was any question of death by the end of 48 hours were examined for the presence of hemorrhage which is typical of an endotoxic effect. The LD_{50} was found to be 8.7 µg with upper and lower 95% embryo confidence limits of 14.0 µg and 5.8 µg respectively.

Table 5. Lethality studies in mice and det. of LD_{50} of a 40-P preparation from P. multocida P-1059 Gray[a]

Dose of toxin (µg)	Dead at no. hours 9	24	48	No. dead / No. challenged	% Dead
500	3	8	4	15/16	93.75
250	1	6	2	9/16	56.25
162.5	0	6	0	6/16	37.5
83.0	0	1	1	2/16	12.5
41.5	0	1	0	1/16	6.25

LD_{50} = 195 µg (148 µg, 265 µg)[b]

[a]Each of the 16-18 gm female mice was inoculated intraperitoneally with the toxin in 0.2 ml of 0.15 M NaCl solution containing 0.3% formalin. The mice were observed for 10 days following inoculation. All mice which died within this period died within 48 hrs.

[b]Values for the LD_{50} and lower and upper 95% confidence limits were determined by probit method of analysis.

Table 6. Lethality studies in chick embryos and determination of the LD_{50} of a P. multocida P-1059 40,000 rpm precipitate[a]

Sample	Toxin dose (µg)	No. dead at 48 hrs. / No. embryos inoculated	% Dead
a	22.0	8/10	80
b	11.0	7/11	63
c	5.5	5/12	42.5
d	2.75	0/10	0
Control	diluent	0/10	0

LD_{50} = 8.7 µg (5.8 µg, 14.0 µg)[b]

[a]The toxin was placed on the chorioallantoic membrane of an artificial air sac; the embryos were observed by candling for 1 week, all embryos which died during this period died within 48 hrs.

[b]Values for LD_{50} and lower and upper 95% confidence limits determined by probit method of analysis.

Chemical Analysis of the Toxic and Immunogenic 40-P Preparations

The immunogenic preparations isolated by centrifugation at 40,000 rpm (105,000 x g) for 2 hours were analyzed for their total nitrogen, phosphorus and carbohydrate content according to the procedures described in Methods. The results of these analyses on a substantial number of preparations are summarized in Table 7. The nitrogen content varied from 4.3% to 8.4%, the carbohydrate content from 13% to 23% and the phosphorus content from 2.0% to 3.67%. In addition to these analyses preparation number 59 was analyzed for heptose using the 3 minute heating procedure of Dische (1955) and found to contain 2.0% (w/w) heptose expressed as α-D-glucoheptose equivalents. Preparation number 30 was analyzed for hexosamine and found to contain 8.1% (w/w) hexosamine using glucosamine as a standard in the procedure of Rondle and Morgan (1955).

Analysis for monosaccharides

Portions of the 40-P preparation were hydrolyzed in sulfuric acid using the conditions described in Methods. The sulfate ion was removed by the addition of $Ba(OH)_2$ and the neutralized hydrolysate was spotted and chromatographed on paper. Two monosaccharides were detected with an alkaline $AgNO_3$ reagent and the location of the spots corresponded to those of galactose and glucose which were chromatographed

Table 7. The chemical analysis of the toxic and immunogenic 40-P preparations obtained from P. multocida P-1059 Gray

Preparation	% nitrogen[a] (w/w)	% carbohydrate[b] (w/w)	% phosphorus[c] (w/w)
Lot 30	6.2	15.5	2.1
Lot 59	5.7	13.5	---
Lot 80	4.26	19.4	2.0
Lot 88	5.0	13.0	---
Lot 109	---	15.9	2.75
Lot 116	4.36	17.8	---
Lot 196	5.1	16.0	2.41
Lot 197	5.6	14.1	2.39
Lot 198	8.4	13.0	2.56
Lot 199	6.35	22.9	3.67
Lot 200	7.1	17.5	2.84

[a]Nitrogen values were determined by microkjeldahl procedure (Kabat and Mayer, 1961).

[b]Carbohydrate expressed as glucose equivalents in the phenol-sulfuric acid procedure (Dubois et al., 1956).

[c]Phosphorus determination by the procedure of Dryer et al. (1957).

simultaneously. The best resolution of these components on paper chromatography was achieved by descending development with butanol-pyridine-water (6:4:3) for 43 hours. Only these two components were observed even when different conditions of hydrolysis were used.

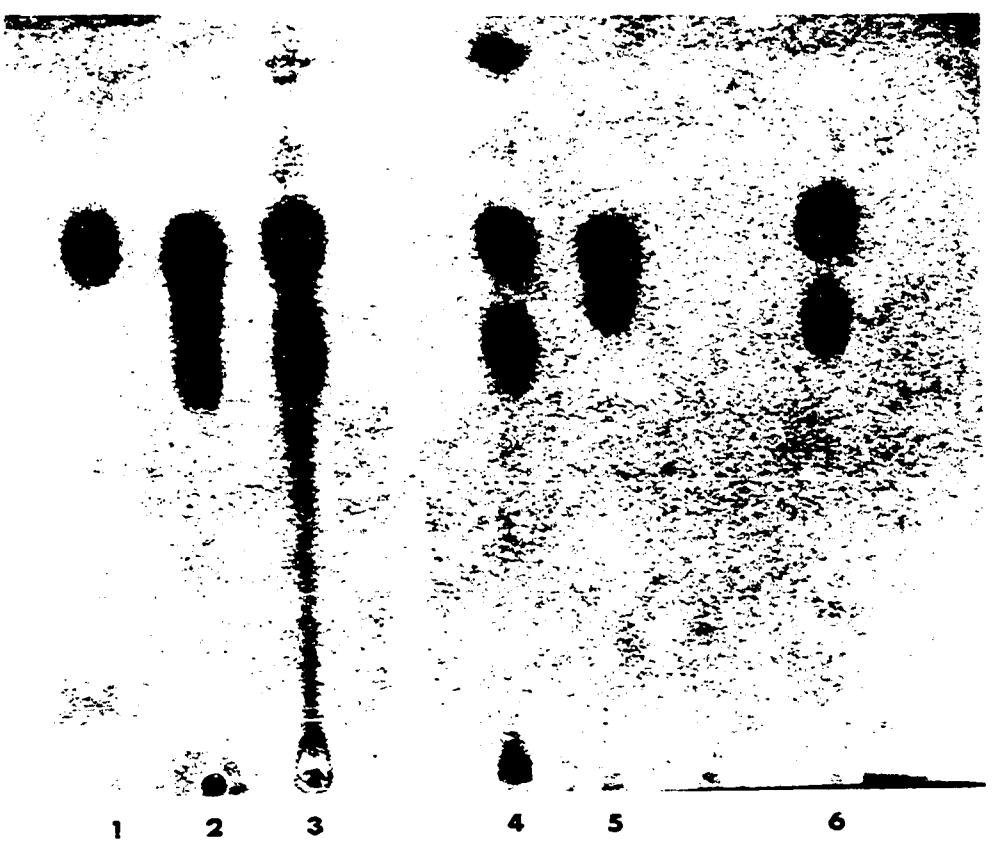
The chromatography of hydrolyzates was also performed on ITLC-SG thin layer chromatograms previously washed with 1% NaH_2PO_4. The same two major components were observed when the developed chromatograms were sprayed with a p-anisidine reagent (Plate 2). However, some additional faint spots were observed and one of these which migrated slightly faster than galactose gave a distinctive reddish-purple color which was also characteristic for heptose standards which were chromatographed simultaneously. This component migrated slower than glucose and slightly slower than α-D-glucoheptose. None of the heptose standards gave the gray-green colors reported to be obtained with p-anisidine-HCl reagent when used on paper chromatograms (MacLennan and Davies, 1957). The best resolution of the monosaccharides was obtained using two developments with acetone-butanol-water (4:5:1). However, the sugars were also resolved with a $CHCl_3$-MeOH-pyridine-water solvent (130:40:1:5) using three developments.

One of the other minor components (faint spot under the conditions used) on the thin layer chromatogram may be a dideoxyhexose, as it had approximately the same mobility as a

Plate 2. Thin layer chromatogram of sugars, an acid hydrolyzate of a P. multocida P-1059 40-P immunogenic preparation and an acid hydrolyzate of a commercial preparation of E. coli lipopolysaccharide

The samples were applied to prepared sheets of ITLC-SG (Gelman Inst. Co.) which had been previously washed with 1% NaH_2PO_4. Sheets developed twice for 30 minutes in acetone-butanol-H_2O (4:5:1). The sugars were located by a p-anisidine reagent.

Samples applied were as follows: 1, glucose; 2, a mixture of glucose, α-D-glucoheptose and galactose; 3, an acid hydrolyzate of the 40-P P. multocida preparation; 4, an acid hydrolyzate of E. coli 0111:B4 lipopolysaccharide; 5, glucose and α-D-glucoheptose; 6, glucose and galactose.


1
2
3
4
5
6

sugar in the E. coli 0111:B4 lipopolysaccharide which is presumed to be colitose (Lüderitz, 1966).

The presence of galactose and glucose in the hydrolyzates was further confirmed by the use of enzymatic assays with Galactostat and Glucostat reagents. The ratio of glucose to galactose was approximately 1.22 to 1.00.

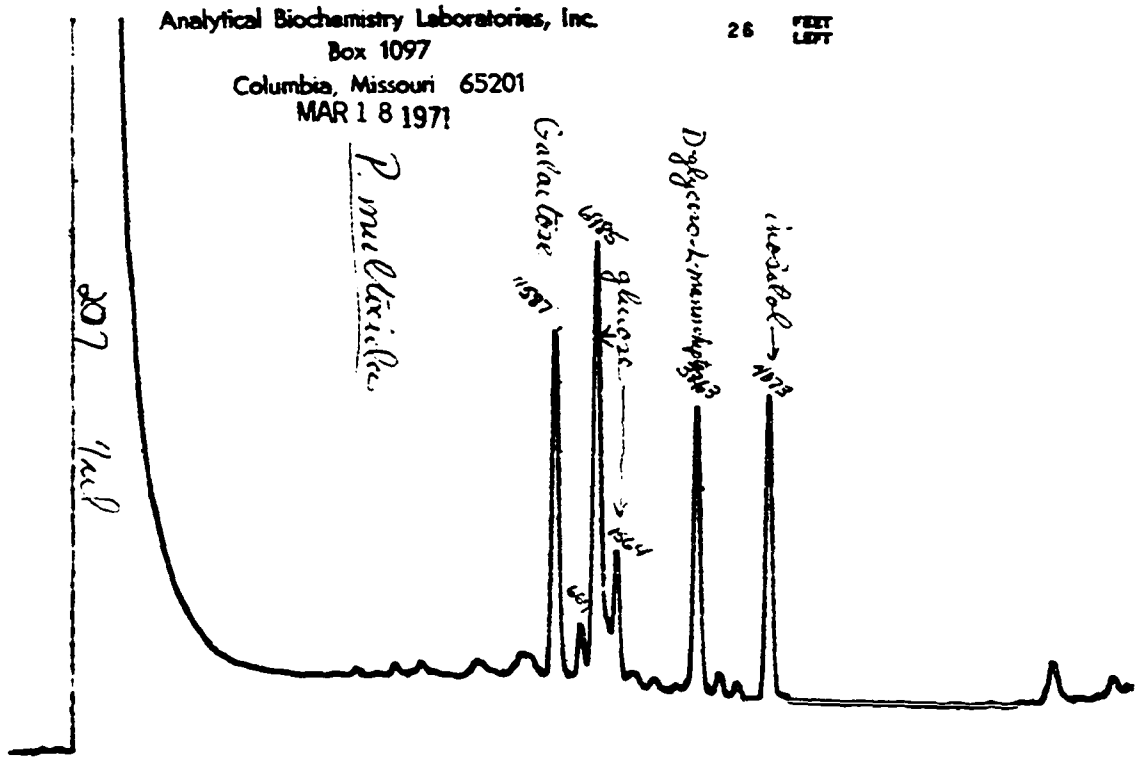
The presence of heptose in the 40-P preparations was also indicated by the characteristic absorption peak at 505 mm for heptoses in the cysteine-sulfuric acid method (Dische, 1955) as mentioned previously. An example of the spectra obtained from this analysis of a 40-P preparation is shown in a following section where it is compared to the spectra obtained from two gel filtration fractions. It is not possible to quantitate the heptose content until the heptose is identified for various heptose sugars have different extinction coefficients in the cysteine-sulfuric acid reaction.

A portion of a dialyzed and lypholized 40-P preparation (lot 109) was sent to Analytical BioChemistry Laboratories, Inc. (Columbia, Mo.) and analyzed by them for the sugar components using gas chromatography. The sugars were first converted to their methyl glycosides by suspending the sample in methanolic 0.5 N HCl and carrying out methanolysis at 80°C for 48 hrs. After removing the lipids by extraction with hexane, and the HCl by repeated evaporation, the solution was passed through a cation exchange resin. The components in

the evaporated solution were then converted into their Sugar-O-trimethylsilyl (TMS) derivatives and analyzed on a gas chromatograph. The above methanolysis conditions are those described by Wang et al. (1970). Three sugars were identified on the gas chromatographs of the P. multocida P-1059 preparations based on their retention times. They were D-glucose, D-galactose and D-glycero-L-mannoheptose. These three sugars were used as external standards and inositol was used as an internal standard. An example of a gas chromatograph is shown in Plate 3a. Three prominent peaks can be seen on the plate in addition to the peak produced by the internal standard inositol. The first and second prominent peaks (left to right) had the same retention time as galactose and glucose respectively. The third prominent peak had the same retention time as a preparation of D-glycero-L-mannoheptose which had been carried through the methanolysis procedure in the same manner as the sample and the glucose, galactose, and inositol standards. A similar pattern of peaks was observed when the samples were chromatographed on 3% OV-1 columns and on 3% SE-30 columns. Although quantitative determinations were not performed, it appears that the ratio of glucose to galactose is in agreement with earlier determinations with Glucostat and Galactostat reagents.

Plate 3a. The analysis of the monosaccharide components of a 40-P preparation (lot 109) as determined by gas chromatography of the trimethylsilyl derivatives of the methyl glycosides

The first large peak (from left to right) had the same retention time as D-galactose, the second the same as D-glucose. The third large peak had the same retention time as D-glycero-L-manno-heptose while the last prominent peak is the internal standard, inositol. Samples were chromatographed on a 3% OV-1 column.


Analytical Biochemistry Laboratories, Inc.
Box 1097
Columbia, Missouri 65201
MAR 1 8 1971
26 FEET LEFT
P. multocida
Galactose
Mannitol
207 Vol

Amino acid analysis

Acid hydrolysates as well as Pronase digests of the 40-P material were examined for amino acids by paper chromatography. The acid hydrolysates gave at least eleven ninhydrin positive components, the most intensely reactive spots appeared to correspond to leucine, alanine, aspartic acid, glutamic acid and glycine.

Pronase digestion of the 40-P preparation, as outlined in the methods section, resulted in paper chromatograms with only a small number of faintly ninhydrin positive components. These appeared to originate from Pronase itself, as the chromatograph of the Pronase control had the same faint components. The activity of the Pronase was confirmed by the proteolysis of casein and paper chromatography of the digest, which showed a large number of ninhydrin positive components.

Evidence that the 40-P preparation was resistent to Pronase action and not merely inhibiting the Pronase activity was obtained by pre-incubation of the 40-P preparation with Pronase followed by the addition of casein and chromatographying the digest on paper. The chromatogram of this digest also showed the presence of a large number of amino acids.

A lypholized sample of a 40-P preparation (lot 109) was sent to Analytical Biochemical Laboratories, Inc., Columbia, Mo., and analyzed for amino acids using gas chromatography. The amino acid content of this preparation is shown in Table 8.

Table 8. The amino acid composition of an immunogenic 40-P preparation isolated from P. multocida P-1059 Gray[a]

Amino acids	Per cent, w/w	Amino acids	Per cent, w/w
Alanine	1.96	Hydroxyproline	0.07
Valine	1.87	Phenylalanine	1.38
Glycine	2.06	Aspartic acid	2.91
Isoleucine	1.20	Glutamic acid	3.69
Leucine	2.34	Tyrosine	1.23
Proline	1.10	Lysine	2.29
Threonine	1.55	Histidine	0.45
Serine	1.61	Arginine	1.53
Methionine	0.35	Cystine/2	0.05
		Total	27.64

[a]The sample was analyzed using the services of Analytical Biochemistry Laboratories, Inc., Columbia, Mo. Analysis was by gas chromatography of samples hydrolyzed in 6N HCl for 4 hours at 145°C using procedure of Gehrke (1970) where N-trifluoroacetyl n-butyl ester derivatives were chromatographed on columns of 0.65% EGA on 80/100 mesh acid washed Chromosorb W and 3% OV-17 on 80/100 mesh Chromosorb G.

The amino acids were released by hydrolysis of the sample in 6 N HCl at 145°C for 4 hours. The amino acid N-trifluoroacetyl n-butyl esters were analyzed on columns of 0.65% w/w EGA on 80/100 mesh acid washed chromosorb W and on 3% w/w OV-17 on 80/100 mesh H. P. Chromosorb G. The amino acids found in highest concentration (w/w) were glutamic acid (3.69%), aspartic acid (2.91%), leucine (2.34%), lysine (2.29%) and

glycine (2.06%).

Lipid analysis

The fatty acid ester content of the extractable and non-extractable lipids from two different 40-P preparations was determined using the procedure of Snyder and Stephens (1959). A 40-P preparation isolated from cells grown on the Proteose Peptone no. 3 agar medium was found to contain 0.72 micro-equivalents of fatty acid ester per milligram dry weight of the sample. A 40-P preparation isolated from a culture grown in Adams-Roe liquid media was analyzed and found to contain 0.91 microequivalents per milligram of sample. Fifty-five percent of the total fatty acid ester content was found to be extractable with $CHCl_3$-MeOH (2:1).

The lipids extracted were analyzed by thin-layer chromatography. The major component (i.e. most intense spot revealed by sulfuric acid charring) was found to migrate at the same rate as a reference preparation of phosphatidyl ethanolamine. This same spot gave a positive reaction with a phospholipid spray reagent and with ninhydrin. No other spots were observed with either the phospholipid detecting reagent or ninhydrin. However, sulfuric acid charring of the chromatograms revealed four other faint spots, three of which migrated faster than phosphatidyl ethanolmine and one slower. In addition to phosphatidyl ethanolamine, phosphatidyl glycerol and phosphatidyl serine were used as references. They both

had a slower migration rate than phosphatidyl ethanolamine and did not correspond to any of the spots seen on sulfuric acid charred chromatograms.

Since the extractable lipid represented 55% of the total fatty acid ester content, and this extract appeared to consist primarily of phosphatidyl ethanolamine, the total extractable phospholipid content could be approximated. Assuming a phospholipid molecular weight of 700 and 2 equivalents of fatty acid ester per mole of phospholipid, the phospholipid (i.e. phosphatidyl ethanolamine) content of the 40-P preparation with 0.72 μ eq./mg would be 12.8% w/w and the other 40-P preparation (preparation from cells grown on Adams-Roe media containing 0.91 μ eq./mg) would have a phospholipid content of 17.7% w/w.

Several attempts were made to hydrolyze the 40-P preparations in order to release lipid A, a component of lipopolysaccharides, but chromatographs of the chloroform soluble fractions on silica gel thin layer plates only resulted in a long broad smear when the plates were charred. The developing solvent was $CHCl_3$:MeOH:H_2O (65:25:4) described by Kasai, 1966.

A 40-P preparation was analyzed through the services of Analytical BioChemistry Laboratories for its fatty acid content by gas chromatography of the fatty acid methyl esters. The methanolysis conditions are the same as those of the sugar analysis described previously and also described by Wang et al. (1970). The results of this analysis are shown in Table 9.

Table 9. The fatty acid analysis of an immunogenic 40-P preparation isolated from P. multocida P-1059 Gray[a]

Fatty acid	Carbon atoms:double bonds	Per cent, w/w
Lauric	12:0	0.42
Myristic	14:0	5.60
Palmitic	16:0	3.85
Palmitoleic	16:1	2.84
Stearic	18:0	0.19
Oleic	18:1	0.50
Linoleic	18:2	trace
Linolenic	18:3	0.07
Arachidic	20:0	trace
Eicosenoic	20:1	trace
Arachidonic	20:4	0.07
	Total	13.54

[a]Analysis obtained through the services of Analytical BioChemistry Laboratories, Inc. The fatty acid content was determined by gas chromatography of the methyl esters obtained from methanolysis in methanolic HCl using procedure described by Wang et al. (1970). Methyl esters were chromatographed on columns of 15% DEGS coated on 80/90 mesh Anakrom ABS and on 10% EGA columns coated on 80/100 mesh acid washed Chromosorb W.

The fatty acids found in highest concentration were myristic (5.60% w/w), palmitic (3.85%) and palmitoleic (2.84%). In addition, there were smaller amounts of oleic (0.50%), lauric (0.42%) and stearic acid (0.19%). There was also a fairly large unidentified peak which had a much longer retention time

on the columns which might correspond to β-OH-myristic acid which has been routinely found in E. coli lipopolysaccharides as reported in a review by Nowotony (1969). Columns of 15% DEGS coated on 80/90 mesh Anakron ABS and 10% EGA coated on 80/100 mesh acid washed Chromosorb W were used.

Isoelectric Focusing Experiments with the 40-P Preparations

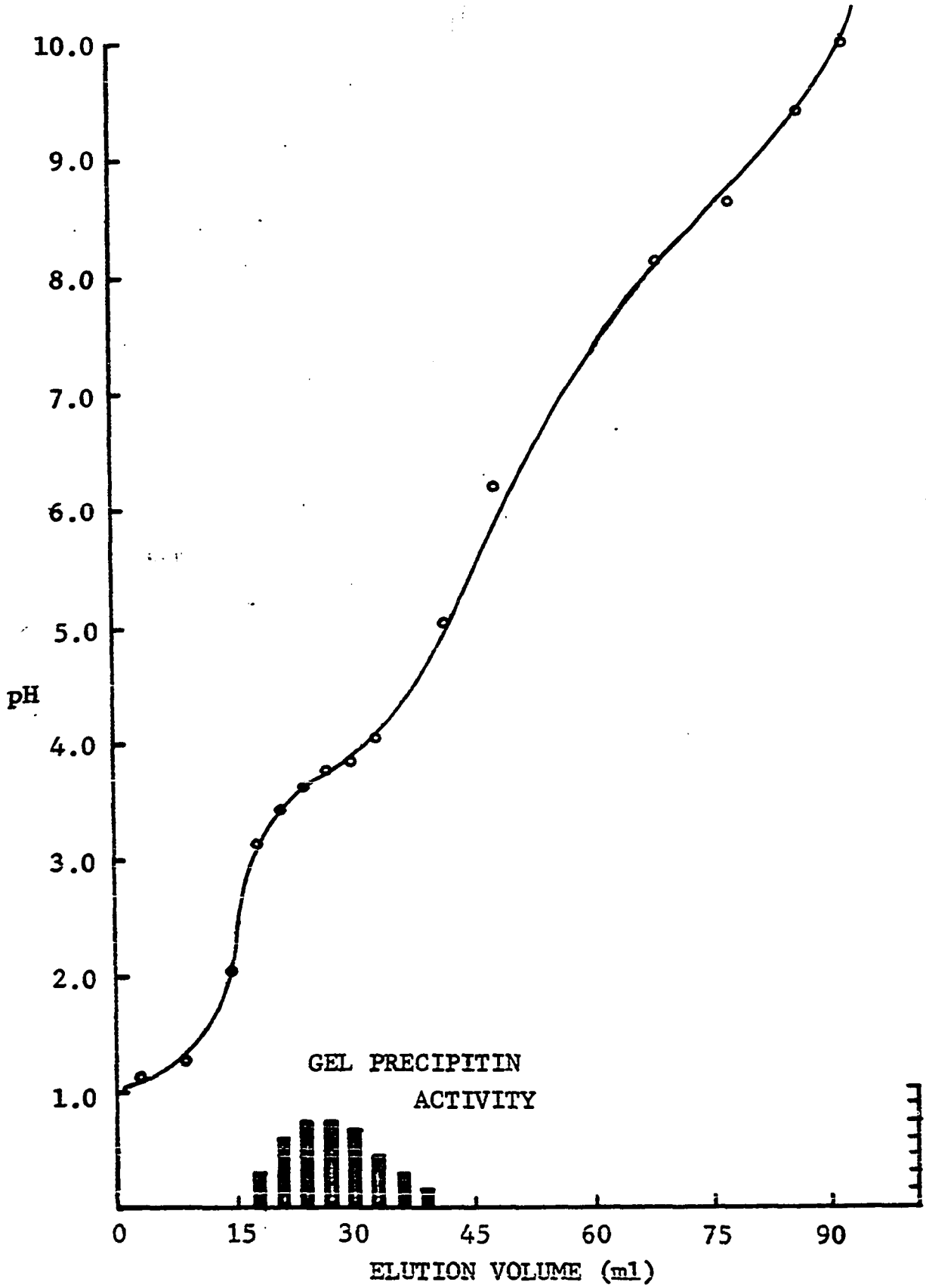
The P. multocida toxic and immunogenic 40-P preparations isolated by high speed centrifugation (105,000 x g, 2 hrs) were studied in a series of experiments using the isoelectric focusing apparatus designed by LKB-Produckter AB. The results of an experiment using a pH 3-10 Ampholine pH gradient and electrofocusing for 20 hours at 300 volts, and 2°C are shown in Figure 1. Each three ml fraction was examined for its serological reactivity in Ouchterlony gel diffusion plates using rabbit antisera to the original 40-P preparations.

Only one region of the gradient gave a precipitin line in the gel diffusion plates. This region or area extended from approximately pH 3.0 to pH 4.5. The distribution of this serological reactivity was nearly symmetrical as estimated by measuring the distance of precipitin lines from the antigen well (an estimate of antigen concentration). Only one precipitin line was observed in the active fractions and the lines from adjacent wells fused with one another indicating serological identity.

Figure 1. An isoelectric focusing experiment with a *P. multocida* P-1059 40-P preparation in a pH 3-10 gradient

Lot number 200 (10 mg) was electrofocused for 20 hours at 300 volts and 2°C.

Solid line represents the pH gradient of the eluted fractions. Solid bars represent the relative concentration of serological reactivity of the fractions as estimated by measuring distance of precipitin lines from the antigen well in Ouchterlony plates.


10.0
9.0
8.0
7.0
6.0
5.0
pH
4.0
3.0
2.0
1.0
GEL PRECIPITIN
ACTIVITY
0
15
30
45
60
75
90
ELUTION VOLUME (ml)

The most intense precipitin line was observed with the reaction of antisera and a fraction which had a pH of 3.7. When the serologically active fractions were pooled, concentrated and reacted with antisera in gel diffusion plates, two precipitin lines were observed which appeared to be identical with the precipitin components of the original 40-P preparation.

Since the isoelectric point of the serologically active material was approximately 3.7, isoelectric focusing experiments were also performed with pH 3-5 gradients in hopes of a more accurate determination of the isoelectric point. However, the 40-P preparations precipitated as soon as they were added to the pH 3-5 Ampholine solutions. Thus, the pH 3-5 gradient experiments failed to give an accurate determination of the isoelectric point, since the precipitate settled to the bottom of the column in the vicinity of the anode. Previous experience with the 40-P preparations had indicated they were insoluble when placed in 0.1 M sodium acetate buffer at pH 4.0.

Electron micrographs of the pooled, dialyzed and concentrated serologically active fractions from the pH 3-10 gradient were taken using negative staining. These electron micrographs indicated the P. multocida preparation consisted of a large number of nearly spherical particles or vesicles. The electron micrographs of these preparations were almost indistinguishable from those taken of Sepharose 2B fractions

of the 40-P preparations. Electron micrographs of these fractions are described in a later section.

Fractionation by Gel Filtration Chromatography

Preliminary experiments

In early experiments of this investigation attempts were made to fractionate the 40-P preparations by Sephadex gel filtration. However, most of the material in the sediment obtained by centrifugation at 105,000 x g for 2 hours was found to pass through Sephadex G-50 and G-200 columns with the void volume. An attempt was made to fractionate these preparations with an agarose column. The column was prepared by mincing a 2% agarose in a blender and sieving it through a series of screens. This agarose column appeared to fractionate the preparations, however, the low recovery of the sample applied (54% of the total nitrogen and 76.5% of the carbohydrate) discouraged the further use of this column.

In recent years preparations of agarose gel particles in a beaded form have come into use for the fractionation and purification of very large macro-molecules, viruses or subcellular particles. Agarose gels of this type, Sepharose 2B, a 2% agarose gel and Sepharose 4B, a 4% agarose gel were used in attempts to isolate, fractionate and characterize the immunogenic and toxic components of 40-P preparations extracted from or liberated by P. multocida strain P-1059.

The first experiments were performed using a 38 x 2.5 cm column of Sepharose 4B. The major amount of the 40-P substance eluted from the column very close to the void volume and the elution of protein, carbohydrate and immunodiffusion precipitins were all found in these same fractions. Since the components were eluted so close to the void volume with Sepharose 4B, experiments were next carried out using Sepharose 2B which has a molecular weight exclusion limit of approximately 50 million compared to the reported exclusion of 15 million for Sepharose 4B (values reported by Pharmacia Fine Chemicals).

Results of Sepharose 2B fractionation

The initial experiments with a Sepharose 2B column (41 x 2.5 cm) were successful in separating the 40-P preparations into three components. A typical elution pattern is shown in Figure 2. A component was eluted at the void volume which hereafter will be referred to as S-I, a second component was eluted near the middle of the separating volume (S-II) and a third component (S-III) was eluted at the position representing the total internal volume, measured by the elution of formaldehyde.

The same column was used then for all subsequent experiments and 2 or 3 ml of different concentrations of the 40-P preparations were applied in each run. Figure 2 also shows the elution profile of Blue Dextran 2000 which was applied to

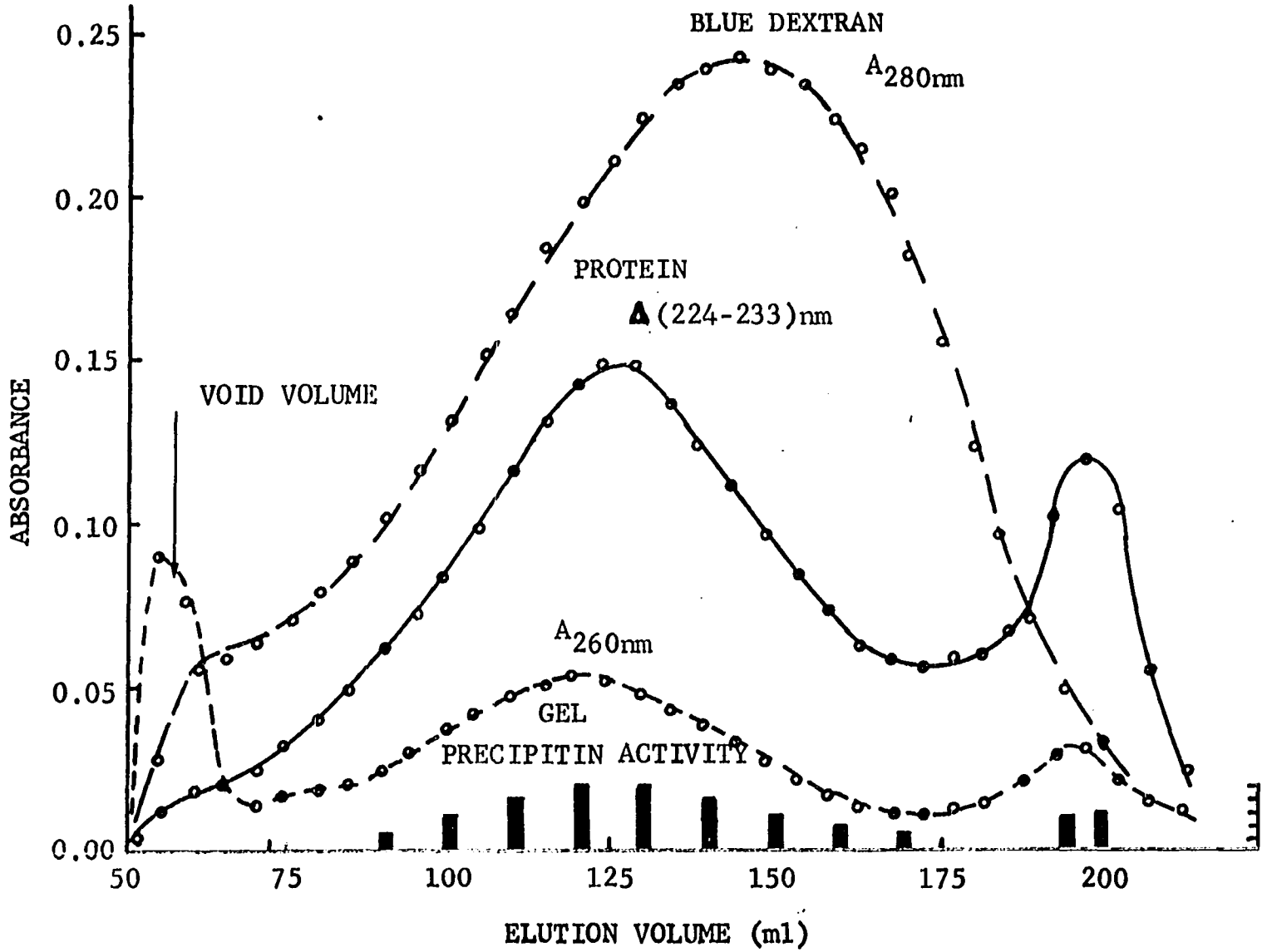
Figure 2. Sepharose 2B gel filtration elution pattern of a P. multocida P-1059 immunogenic 40-P preparation and a Blue Dextran preparation chromatographed separately on the same 41 x 2.5 cm column. 4.2 mg of a 40-P preparation (lot #226) in 2 ml was applied to the top of the column and eluted with 0.15 M NaCl containing 3% ethanol using a constant flow rate of 2.24 ml/cm^2/hr. and collecting 5 ml fractions

o--o--o upper dashed curve is elution pattern of Blue Dextran

o—o—o solid curve represents the protein elution curve of the 40-P immunogenic preparation

o--o--o lower dashed curve represents the 260 nm absorption curve of the 40-P immunogen

Bars represent the presence of precipition lines in gel diffusion plates and their height is proportional to the distance of line from edge of antigen well.


BLUE DEXTRAN
A_{280nm}
PROTEIN
Δ(224-233)nm
VOID VOLUME
A_{260nm}
GEL
PRECIPITIN ACTIVITY
ABSORBANCE
0.25
0.20
0.15
0.10
0.05
0.00
50
75
100
125
150
175
200
ELUTION VOLUME (ml)

and eluted from the same column periodically to check the void volume. For this column the void volume was 57.5 ml and appeared constant throughout the following series of experiments. Unless noted otherwise, the eluant in all experiments was 0.85% NaCl containing 3% v/v ethanol, pH 5.5 and the flow rate of 10-11 ml/hour was maintained by a pressure head of approximately 9 cm through the use of a Mariotte flask. Each 5 ml fraction was examined at the conclusion of the 20-22 hour chromatography for its ultraviolet absorption at four different wavelengths: 224, 233, 260 and 280 nanometers (nm). The 224 and 233 nm wavelengths were useful in locating the peak elution volumes of the various components, especially those present in trace amounts. In addition the absorption difference between 224 and 233 nm ($\Delta A_{224-233\ nm}$) was useful in quantitating the amount of eluted protein according to the method of Groves et al. (1968). This method can be used to determine protein in the presence of nucleic acid.

Since 16 different gel filtration experiments were performed with 11 different P. multocida P-1059 40-P preparations, it is not possible or practical to describe the results of all these experiments here. For this reason only the results of a representative number of experiments will be described. These experiments point out some of the differences and similarities of the elution patterns obtained. As mentioned above the eluted fractions were examined with a spectrophotometer for

ultraviolet absorption at four different wavelengths. The two or three peaks observed in the majority of the preparations were all found to occur at the same position regardless of which wavelength was used to plot the elution profiles. Thus the reason for plotting the elution profiles with different ultraviolet wavelengths in the following figures is strictly a graphical one. The lower wavelengths 224 and 233 nm were more effective for detection when small amounts of sample were applied to the column and the higher wavelengths 260 and 280 nm could be used when larger samples were applied.

The following paragraphs describe the general nature and relative amounts of the material in the three peaks designated S-I, S-II and S-III. Following this is a description of specific experiments where either the carbohydrate and protein distributions are compared or the quantity of sample applied to the column was varied.

Component S-I which is eluted at the void volume (see Figure 2), contained a small amount of material which may be nucleic acid or nucleoprotein based on its absorption at 260 nm. In the 16 different gel filtration experiments the component S-I never amounted to more than 5%-10% (w/w) of the eluted sample. In many cases it was barely detectable. It did not give a precipitin line in Ouchterlony immunodiffusion plates even when highly concentrated.

The substance with an elution peak from 115-125 ml (S-II) was the major fraction or component in all preparations and accounted for 80-100% of the dry weight of the applied and eluted samples. It accounted for most of the protein, Kjeldahl total nitrogen and carbohydrate of the applied sample. In immunodiffusion plates, this component gave a single precipitin line when reacted with antisera to the whole saline extract of P. multocida strain P-1059. This precipitin line fused with the major precipitin line observed in the original 40-P preparation when the two preparations were placed in adjoining wells, indicating serological identity. Component S-II, however, did give two precipitin lines when either individual or pooled fractions were concentrated to approximately 3 mg/ml and reacted with hyperimmune rabbit antisera to the original 40-P or whole saline extract of P. multocida P-1059. This was also true of the original 40-P preparations in that they gave two slowly diffusing immunoprecipitins when high concentrations were examined in Ouchterlony plates (see for instance Plate 1a).

The component S-III representing the material eluted at a volume of 190-195 ml as shown in Figure 2 was present in several 40-P preparations but the relative amount varied from one preparation to another just as was the case for component S-I. When S-III was present in significant amounts, fractions representing this material gave one and sometimes two precipitin lines in immunodiffusion plates. They, however,

did not fuse with the precipitin lines observed with S-II fractions in an adjacent well.

In several experiments aliquots of the eluted fractions were analyzed for the carbohydrate content by the phenol-sulfuric acid method. An example of one of these experiments is shown in Figure 3. In most cases S-II was the major carbohydrate fraction with varying amounts of carbohydrate in S-I and S-III. When separate portions of the same 40-P preparation were applied to the column, the ultraviolet and carbohydrate elution profiles were exactly the same as far as could be determined. In fact when the same quantity and volume were applied the ultraviolet and carbohydrate elution curves were superimposable with corresponding fractions giving the same absorption ($\pm$ .005).

Although the same elution profile was obtained from separate aliquots of an individual 40-P preparation, the elution profiles did vary from one 40-P preparation to another (see Figure 4). As stated previously the most significant changes were in the relative amounts of S-I and S-III. This fact is obvious when the results of experiments as shown in Figures 3 and 4 are compared. All gel filtration figures represent the elution patterns from different 40-P preparations except Figure 4 which is the same preparation used for Figure 2. Although the amounts of components varied, the elution volumes of the peaks were remarkably consistent. There was, however, a

Figure 3. Sepharose 2B gel filtration of a 12 mg/2 ml aliquot of 40-P preparation (lot #197)

Upper dashed line represents the 280 nm absorption of the fractions.

Lower solid curve represents the 490 nm absorption of the phenol-sulfunic acid analysis for carbohydrate on 0.5 ml aliquots of the 5 ml fractions.

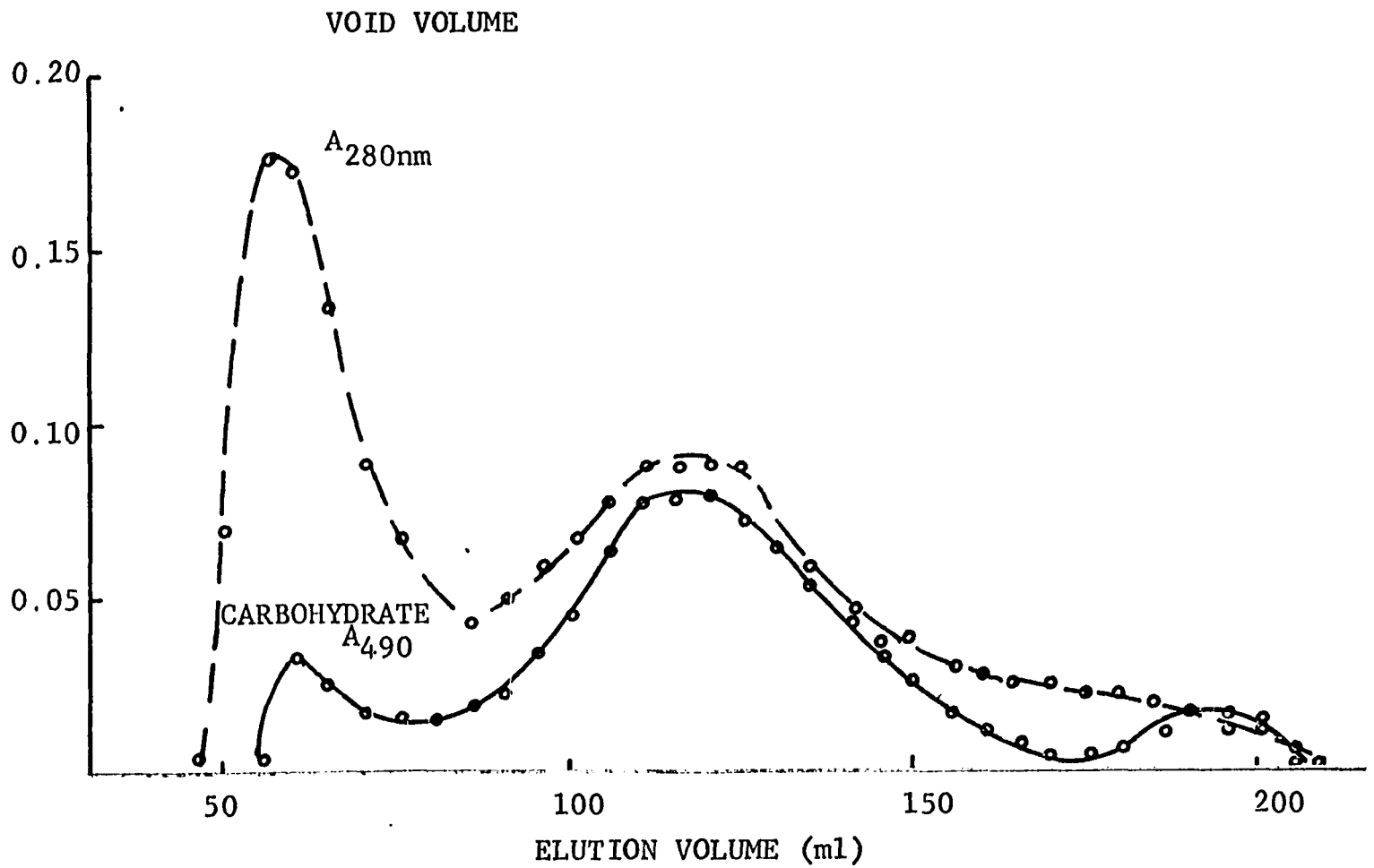
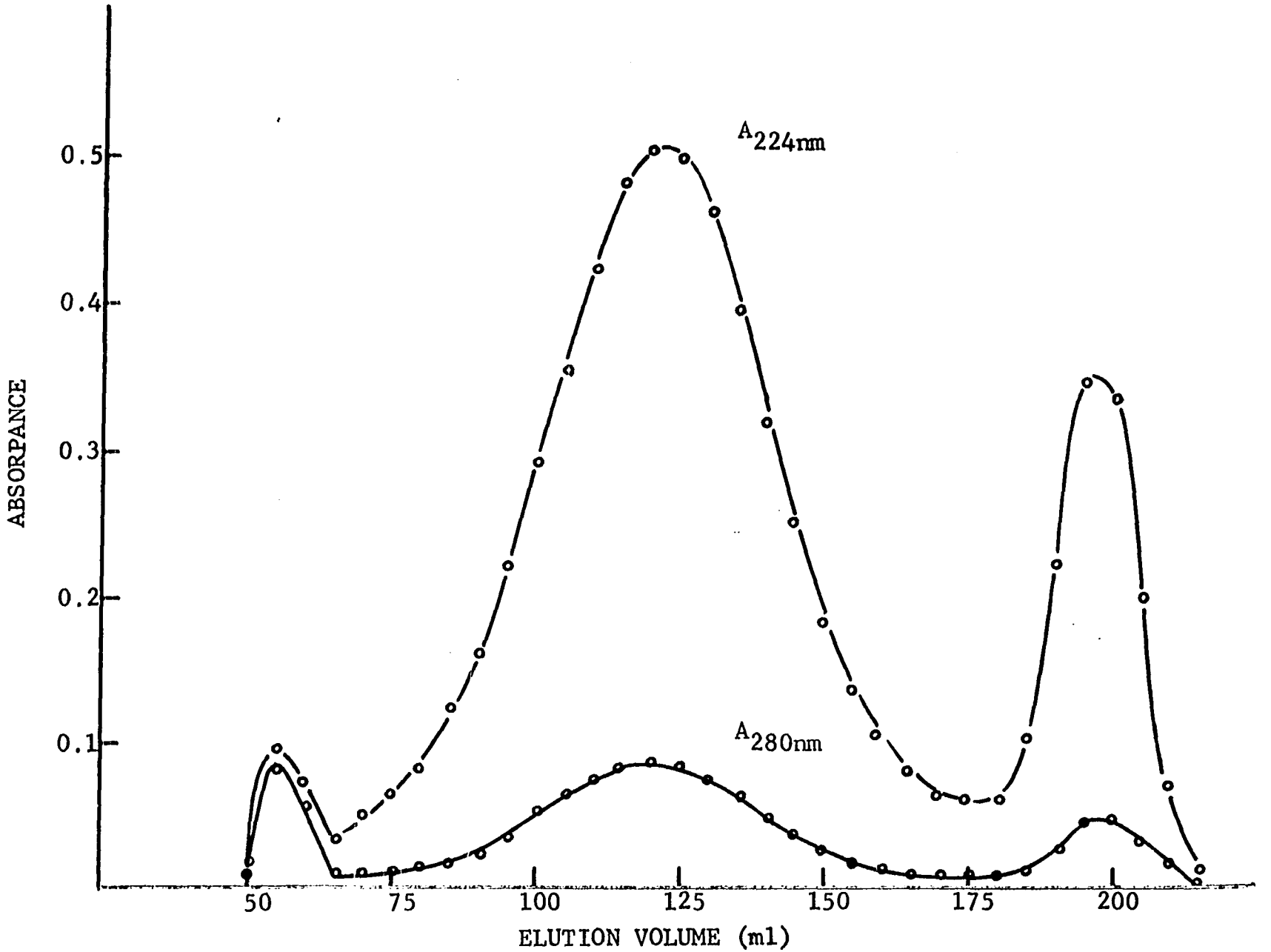

VOID VOLUME
0.20
0.15
0.10
0.05
A_{280nm}
CARBOHYDRATE
A_{490}
50
100
150
200
ELUTION VOLUME (ml)

Figure 4. Sepharose 2B gel filtration of 6.3 mg in 3 ml portion of a 40-P preparation #226

Upper curve represents the 224 nm absorption of each 5 ml fraction.

Lower curve represents the 280 nm absorption of the eluted fractions.

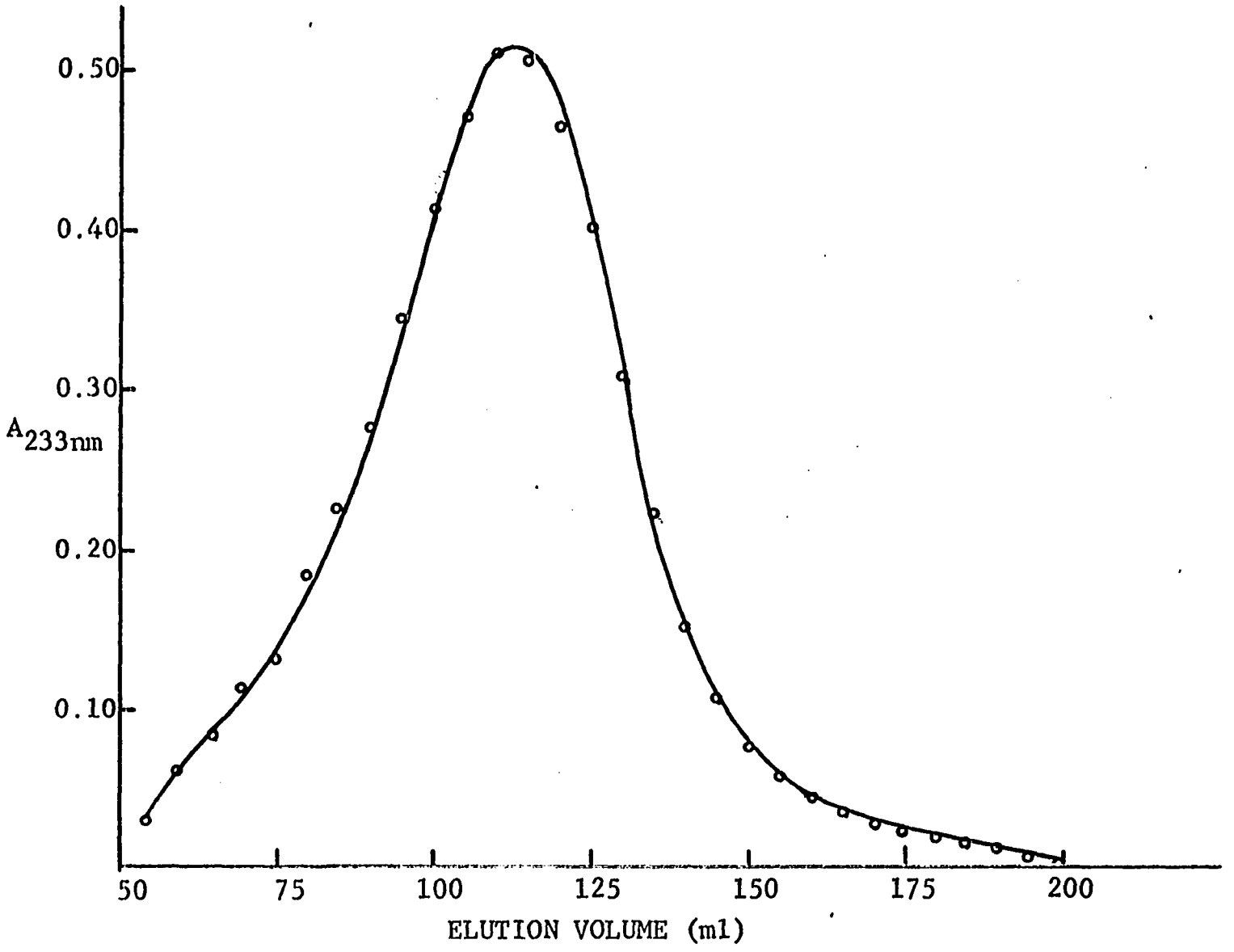

ABSORPANCE
0.5
0.4
0.3
0.2
0.1
A_{224nm}
A_{280nm}
50
75
100
125
150
175
200
ELUTION VOLUME (ml)

slight shift in the elution volume of the major component S-II which appeared to be somewhat concentration dependent. When samples containing 4-5 mg/2 ml were applied, the peak elution volumes of S-II were on the order of 120-125 ml while application of samples containing 12-25 mg/2 ml resulted in S-II elution volumes of 113-120 ml. The amount of 40-P material applied to the column did not appear to effect the position of S-I which represents material found at the void volume or the position of S-III, the low molecular weight material. Neither did the quantity of applied sample appear to effect the symmetry or resolution of the elution peaks unless samples containing at least 25-30 mg in volumes of 4 ml or more were applied when the elution peaks were skewed and less well resolved.

An example of a gel filtration experiment with a larger amount (approximately 20 mg) of applied 40-P sample is shown in Figure 5. This preparation was typical of a few 40-P preparations in that it gave only one newly symmetrical elution peak apparently the same as S-II of other preparations. This experiment was also typical of many experiments in that as mentioned above the application of larger amounts resulted in slightly lower peak elution volumes. In this case the peak elution volume was between 110-115 ml. The ultraviolet absorption at 233 nm is shown on this figure as the 224 nm absorption exceeded 2.0 for part of the curve. Although not

Figure 5. Sepharose 2B gel filtration of a concentrated sample (20 mg/2 ml) of 40-P preparation (lot 200)

Curve represents the 233 nm absorption of the 5 ml fractions.


0.50
0.40
0.30
A_{233nm}
0.20
0.10
50
75
100
125
150
175
200
ELUTION VOLUME (ml)

shown in this figure, the major serological reactivity of the fractions obtained in this experiment and in all others appeared to correspond to the S-II elution curve.

Since experiments described earlier had shown that the three peaks contained carbohydrate as well as protein, a more extensive study was made of the ratio of protein to carbohydrate. In a gel filtration experiment where 21.6 mg was applied to the Sepharose 2B column (see Figure 6), each 5 ml fraction was analyzed for protein by the differential ultraviolet absorption method of Groves et al. (1968) and by the Folin method (Bailey, 1967) using 0.2 ml aliquots. In addition 0.5 ml aliquots of each fraction were analyzed for carbohydrate by the phenol-sulfuric acid method (Dubois et al., 1956).

The absorption values shown in Figure 6 represent the actual observed readings of each analysis. The concentration of protein per ml of solution was calculated for both protein determinations and is shown in Table 10 along with the concentration of carbohydrate per ml expressed as glucose equivalents. The ratio of protein to carbohydrate and calculated separately using the Groves and the Folin method of protein analysis. As shown in Table 10, the protein to carbohydrate ratios were not constant across the entire elution curve for the major component S-II. The protein to carbohydrate ratio of component S-II varied from approximately 3.0/1 (fraction 19) to 2.4/1 (fraction 23) to 2.8/1 (fraction 29) when protein was

Figure 6. Sepharose 2B gel filtration of a 40-P preparation (lot #116) 21.6 mg/3 ml applied using flow rate and eluant as previously described

o—o—o solid curve represents the ΔA(224-233)nm method of determining protein (Groves et al., 1968); 100 µg/ml bovine serum albumin had ΔA of 0.570.

o--o--o dashed curve with solid circles represents the 490 nm absorption in phenol-sulfuric acid determination for carbohydrate (Dubois et al., 1956); 0.5 ml aliquot analyzed; 15 µg glucose had absorption of 0.312.

o-o-o dashed curve with open circles represents the 750 nm absorption of Folin determination (Bailey, 1967) for proteins; 0.2 ml aliquots analyzed; 10.7 µg bovine serum albumin had absorption of 0.168.

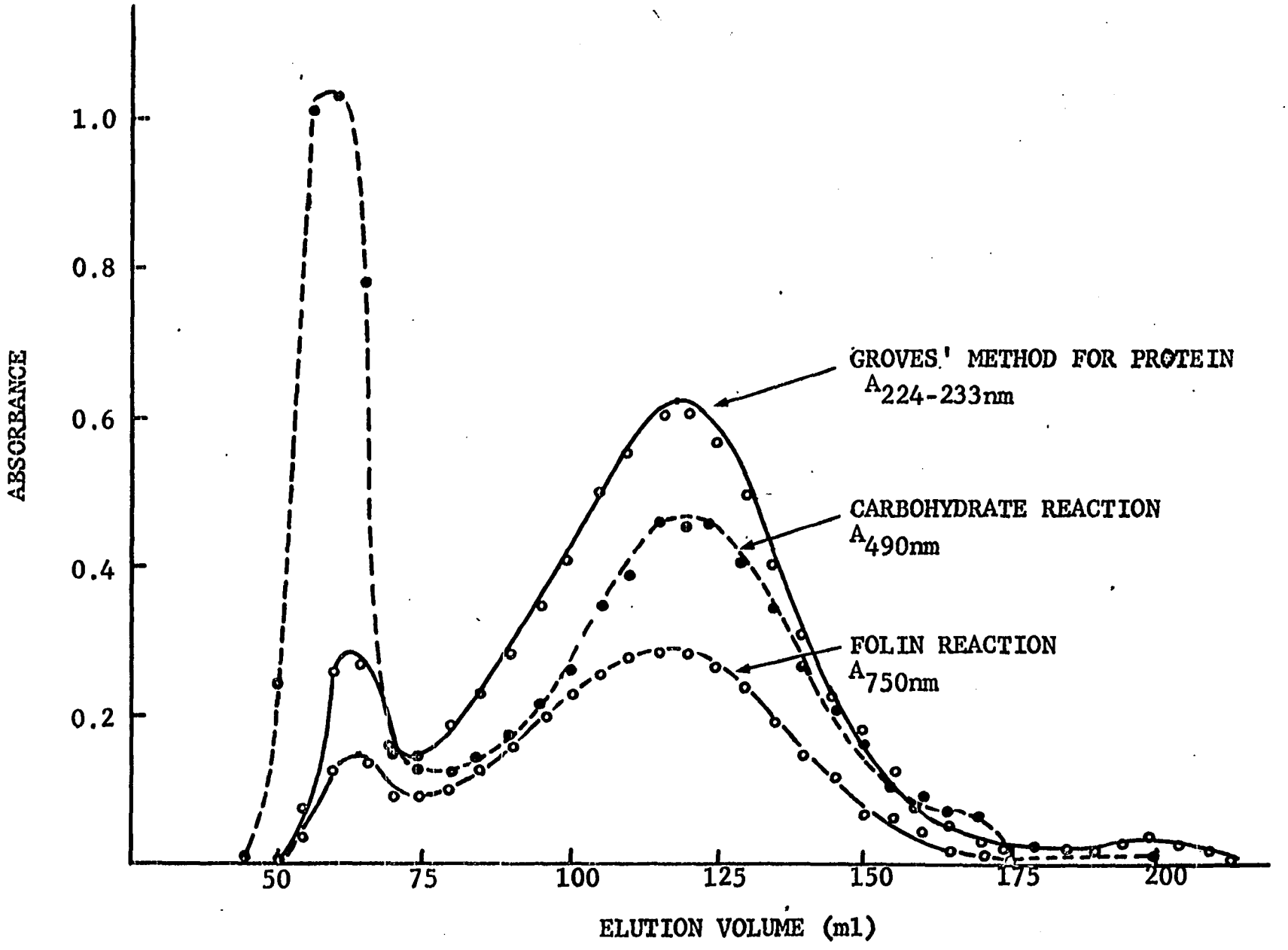

ABSORBANCE
1.0
0.8
0.6
0.4
0.2
50
75
100
125
150
175
200
ELUTION VOLUME (ml)
GROVES' METHOD FOR PROTEIN
$A_{224-233nm}$
CARBOHYDRATE REACTION
A_{490nm}
FOLIN REACTION
A_{750nm}

Table 10. The protein and carbohydrate concentration and the ratio of protein to carbohydrate for each fraction of the experiment whose elution pattern is shown in Figure 6[a]

Fraction number		Protein (method of Groves) Column a µg/ml	Protein Folin method Column b µg/ml	Carbohydrate Phenol-sulfuric Column c µg/ml	Ratio a/c	Ratio b/c
11		13.2	12.8	96.7	.136	.132
12		46.3	45.8	100.5	.461	.456
13	S-I	48.2	47.2	73.1	.659	.646
14		27.9	28.1	14.7	1.90	1.91
15		24.7	29.7	12.5	1.98	2.38
16		34.0	32.8	11.0	3.09	2.98
17		41.0	40.5	12.3	3.33	3.29
18		50.3	49.8	15.2	3.31	3.28
19		61.4	62.2	20.7	2.97	3.00
20		73.7	73.4	24.8	2.97	2.96
21		88.1	82.3	33.0	2.67	2.49
22		98.2	94.8	36.7	2.68	2.58
23		105.8	94.1	43.3	2.44	2.20
24	S-II	106.3	103.7	43.0	2.47	2.41
25		100.3	87.8	42.1	2.38	2.19
26		88.2	77.9	34.0	2.59	2.29
27		71.7	63.2	25.8	2.78	2.45
28		55.4	47.2	20.3	2.73	2.33
29		41.4	36.1	14.6	2.84	2.47
30		29.1	22.7	10.4	2.80	2.18
31		20.2	19.2	8.4	2.41	2.29

[a]Five ml fractions were collected, thus fraction 11 has elution volume of 55 ml and fraction 24 an elution volume of 120 ml, etc.

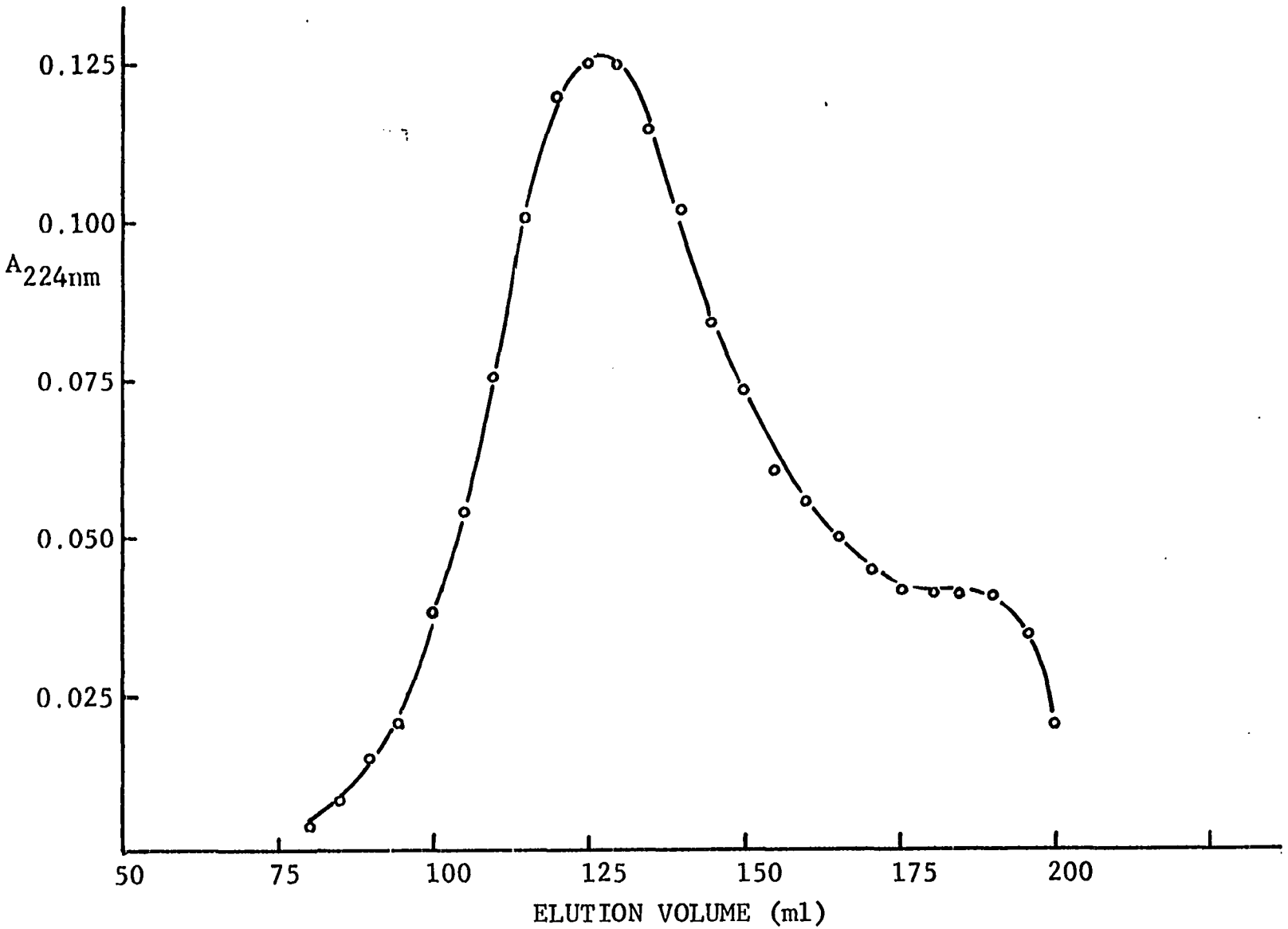
determined by the ultraviolet method and from approximately 3.0/1 (fraction 19) to 2.2/1 (fraction 23) to 2.5/1 (fraction 29) when the protein concentration was determined by the Folin method. There was reasonably good agreement between the Folin and Groves method for determination of protein as can be seen in Table 10. However, the concentration values were in slightly better agreement at the first part of the curve than at the latter.

Since the calculation of the protein to carbohydrate ratio resulted in a variation for portions of the large peak S-II, fractions 26-30 which represent elution volumes of 125-150 ml in the above experiment were pooled, concentrated to 2 ml by a collodion membrane apparatus and re-applied to the same Sepharose 2B column. The elution pattern for this re-cycled sample is shown in Figure 7. Although the 224 nm absorption maximum occurred between 125-130 ml in this experiment as opposed to a 115 ml elution volume in the first cycle, this difference is most likely due to the smaller amount (approximately 4 mg) which was applied to the column. This concentration effect has been described previously.

Each fraction in the main part of the large peak shown in Figure 7 was analyzed for protein by the Groves procedure and for carbohydrate by the phenol sulfuric acid method using the procedures described previously. The data on these fractions and the ratio of protein to carbohydrate is summarized in

Figure 7. Sepharose 2B gel filtration of concentrated pooled fractions representing elution volumes from 125-150 ml of experiment in preceding figure; approximately 1.4 mg protein applied in 2 ml

Curve represents 224 nm absorption of 5 ml fractions.


0.125
0.100
A_{224nm}
0.075
0.050
0.025
50
75
100
125
150
175
200
ELUTION VOLUME (ml)

in Table 11. The same type of variation in the ratios of protein to carbohydrate are evident in this table as in the previous one.

Table 11. Analysis of the protein and carbohydrate content of the S-II fractions as shown in Figure 7, a recycled portion of the fractions from experiment in Figure 6

Fraction number	Protein Groves method Column a µg/ml	Carbohydrate Phenol sulfuric Column b µg/ml	Ratio Col. a/Col. b
22	6.0 ± 1.0	2.1 ± 0.5	2.8
23	8.6 "	3.1 "	2.8
24	11.0 "	4.0 "	2.8
25	12.2 "	4.7 "	2.6
26	12.8 "	4.2 "	3.0
27	12.0 "	4.3 "	2.8
28	11.2 "	3.5 "	3.1
29	9.6 "	2.7 "	3.5

Even though the ratios are not constant for the peak S-II material as might be expected for a chemically homogeneous substance, the actual variation which is shown in the tables could well be explained by the combination of the experimental errors in the different methods of analysis.

The phenol-sulfuric acid method may well be subject to additional errors produced by variable amounts of carbohydrate being released from the agarose column. In earlier experiments it was found that other agarose columns released materials which gave a wave-like elution pattern when fractions were analyzed for total carbohydrate using the phenol-sulfuric acid procedure. The blank elution fractions in these experiments gave absorptions in the carbohydrate analysis which were equivalent to 0.5 to 1.0 μg of glucose equivalents. These amounts could account for at least 10% errors in the carbohydrate concentration.

Since errors in the carbohydrate determination could easily account for a 10% variation in the protein to carbohydrate ratio, and the protein determinations are also subject to error, it is not possible on the basis of present data to determine whether or not there was any real differences in the chemical composition of the leading edge and trailing edge of the main Sepharose 2B fraction S-II.

A duplicate experiment was carried out with the same preparation and using the same conditions as those described in Figure 6. The fractions representing elution volumes of 80 ml-160 ml which includes the main peak S-II were pooled and portions were analyzed in triplicate for microkjeldahl nitrogen. The carbohydrate content of peak S-II was determined by summation of the content of the individual fractions

and the protein content was determined by Groves procedure. The results of the analysis are summarized in Table 12 along with the analysis of the original 40-P preparation.

The results of these analyses are consistent with the results of the chromatographic elution pattern shown in Figure 6. The separation of the peak S-I material which is primarily carbohydrate from the S-II protein-carbohydrate complex is obvious in this experiment.

Although there was a limited amount of the material in peak S-I, which precluded extensive chemical analysis, it was possible to analyze a portion of the sample for heptose in the Dische (1955) cysteine-sulfuric acid procedure. The spectra obtained in this reaction can also be useful in determining the presence of hexoses and other monosaccharides. The absorption at various wavelengths for aliquots of peak S-I, peak S-II, and the original 40-P preparation is shown in Table 13 along with the spectra of a commercial preparation of α-D-glucoheptose.

Dische (1955) reports that in the cysteine-sulfuric acid procedure hexoses in general form a chromogen which absorbs strongly near 410 nm while heptoses form a chromogen which has a maximum absorption near 505 nm. He also reports that hexoses form another chromogen with an absorption maximum near 600 nm and that this chromogen formed by galactose upon standing has a more pronounced absorption than glucose. From these

Table 12. Chemical analysis of the Sepharose 2B peak S-II protein-carbohydrate complex and the original unfractionated 40-P preparation

Sample	Method of analysis			
	MicroKjeldahl[a]		Groves u.v.[b]	Phenol-sulfuric acid[c]
	% N (w/w)	% protein (w/w)	% protein (w/w)	% carbohydrate (w/w)
Sepharose 2B peak S-II	4.10	25.6	27.8	10.7
Original 40-P preparation (lot 116)	4.36	27.3	----	17.8

[a]The Markham microKjeldahl procedure as described by Kabat and Mayer (1961).

[b]Differential ultraviolet absorption method of Groves et al. (1968).

[c]Phenol-sulfuric acid procedure as described by Dubois et al. (1956). Carbohydrate value expressed as glucose equivalents.

observations and the knowledge that the original 40-P preparation contained glucose, galactose and a heptose, it appears that the carbohydrate in peak S-I does not contain the same ratios of the monosaccharides found in the major peak S-II. This is particularly evident when the relative absorptions at 410 nm and 505 nm are compared. From the spectra it appears that peak II contains a heptose in a much higher proportion to the total hexose than peak I.

Table 13. The Dische cysteine-sulfuric acid reaction for heptoses and other sugars on the Sepharose 2B peaks S-I and S-II, the original 40-P preparation and a glucoheptose standard[a]

Wavelength nm	Sample and absorption: Glucoheptose (122 µg) dry weight	Peak S-I (64 µg) carbohydrate[b]	Peak S-II (78.6 µg) carbohydrate	Original 40-P (213 µg) carbohydrate
385	.106	.710	.750	1.530
400	.093	1.03	1.00	2.28
410	.099	1.184	1.144	2.65
440	.143	.474	.509	1.254
480	.494	.092	.225	.553
500	.824	.079	.233	.520
505	.848	.078	.234	.517
510	.838	.075	.226	.508
540	.336	.073	.204	.450
570	.040	.090	.230	.370
590	.034	.112	.270	.337
600	.032	.116	.270	.324
610	.030	.103	.235	.304

[a]The secondary cysteine-sulfuric acid procedure; the absorption of the samples was read at 22 hours following reaction (Dische, 1955).

[b]Carbohydrate concentration determined by phenol-sulfuric acid procedure (Dubois et al., 1956).

Gel filtration using Tris-EDTA

All the previously described experiments of gel filtration of the 40-P preparations on Sepharose 4B and 2B were performed using 0.85% w/v NaCl containing 3% v/v ethanol, pH 5.5 as the eluant. Because the pH and the chelating agent ethylenediamine tetraacetate (EDTA) might be expected to change the physical properties and the size distribution of the 40-P components (see for example, Rogers, 1971), a 0.1 M Tris-HCl buffer pH 8.2 containing 0.01 M Na_2EDTA was also used as an eluant in a series of experiments. The samples of 40-P material were dialyzed 10 days at 4°C in the presence of this buffer and then applied to the same column described previously. There was no apparent effect on the relative elution volumes but the total internal volume of the column appeared to be shortened as shown by the elution volume of formaldehyde and Blue Dextran was found to give a compressed elution curve with Tris buffer as compared to the 3% ethanolic NaCl. This caused a considerable loss in the resolution of the components S-I, S-II and S-III however, the protein and carbohydrate in the fractions appeared to be distributed in the same manner as when NaCl was used as the eluting solution.

Gel filtration of a culture filtrate concentrate

Since the component S-II was consistently found in all 40-P preparations, it was of interest to see if a similar component could be isolated from the original culture filtrate

without the preliminary isolation procedure of repetitive centrifugation at 40,000 rpm. The results of such an experiment are shown in Figure 8. A 300 ml volume of the culture filtrate was concentrated to a small volume (10 ml) with Diaflo XM-100 membranes which reportedly allow globulin proteins of under 100,000 MW to pass through. No attempt was made to remove the proteins under 100,000 MW by repeated concentration. Two ml of this concentrate was applied to a column using the same conditions described for 40-P preparations. Two peaks were observed, the smaller peak contained approximately 6% of the total protein and the larger peak contained approximately 94% when analyzed by the protein method of Groves et al. (1968). The smaller peak had an approximate elution volume of 125 ml and gave a precipitin line in immunodiffusion plates which fused with the line formed by the S-II component of the 40-P preparations found in the adjacent well, indicating serological identity. The large peak in Figure 8 gave 3-4 precipitin lines in the same plates but did not give lines of identity with either the small peak or S-II components of 40-P preparations.

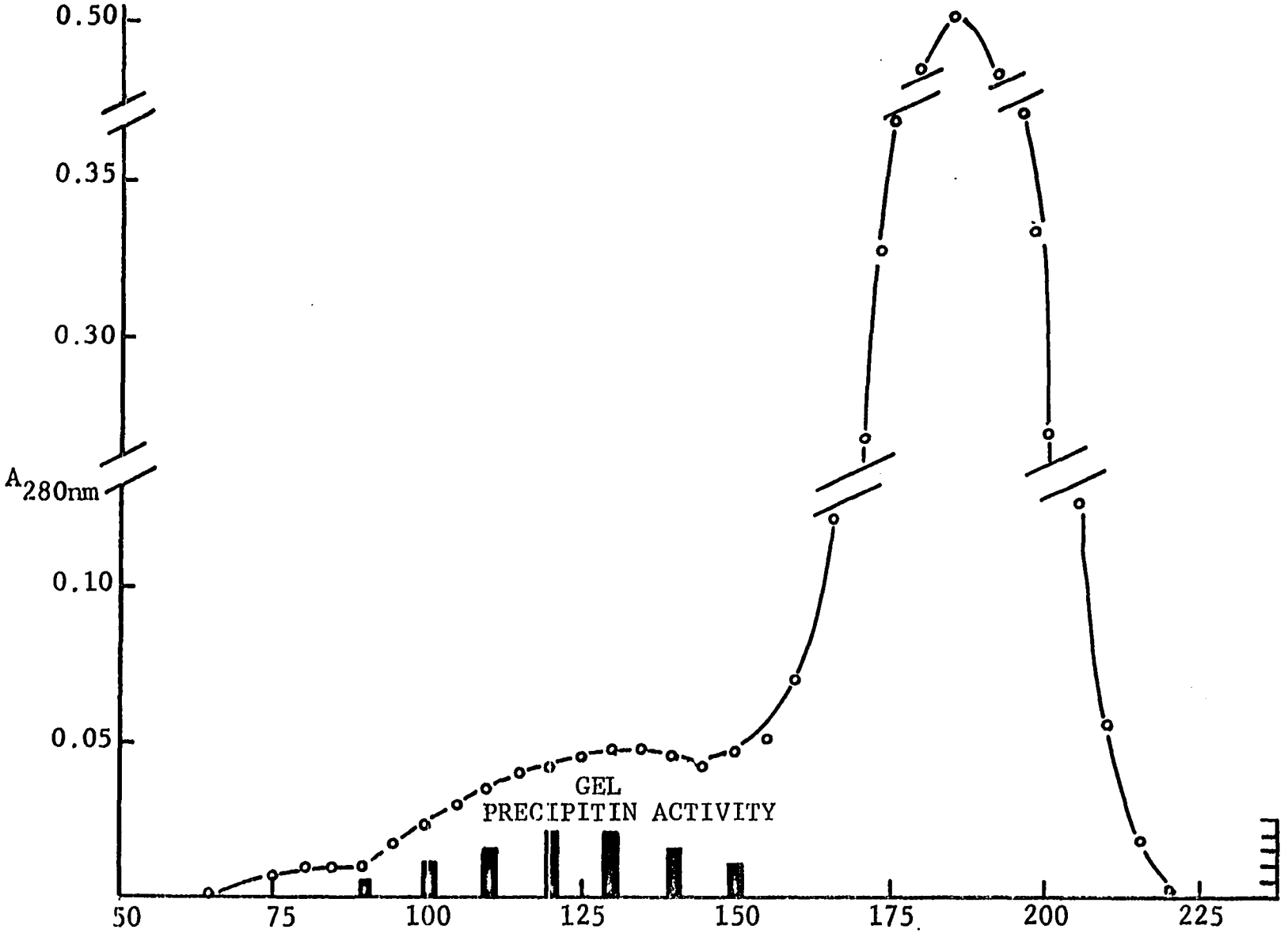
Gel filtration of 80s ribosomes

Numerous attempts were made to obtain macromolecules or biological particles suitable for calibrating and analyzing the resolving ability of the Sepharose 2B column used in these experiments. Suitable reference substances were not available

Figure 8. Sepharose 2B gel filtration of a Diaflo X-100 membrane concentrate of the P. multocida culture filtrate (lot 226)

Curve represents the 280 nm absorption of each 5 ml fraction.

Bars represent the presence of gel precipitin line which appears to be serologically identical with peak II material of 40-P filtration experiments; height of bars represents distance of precipitin line from antigen well.

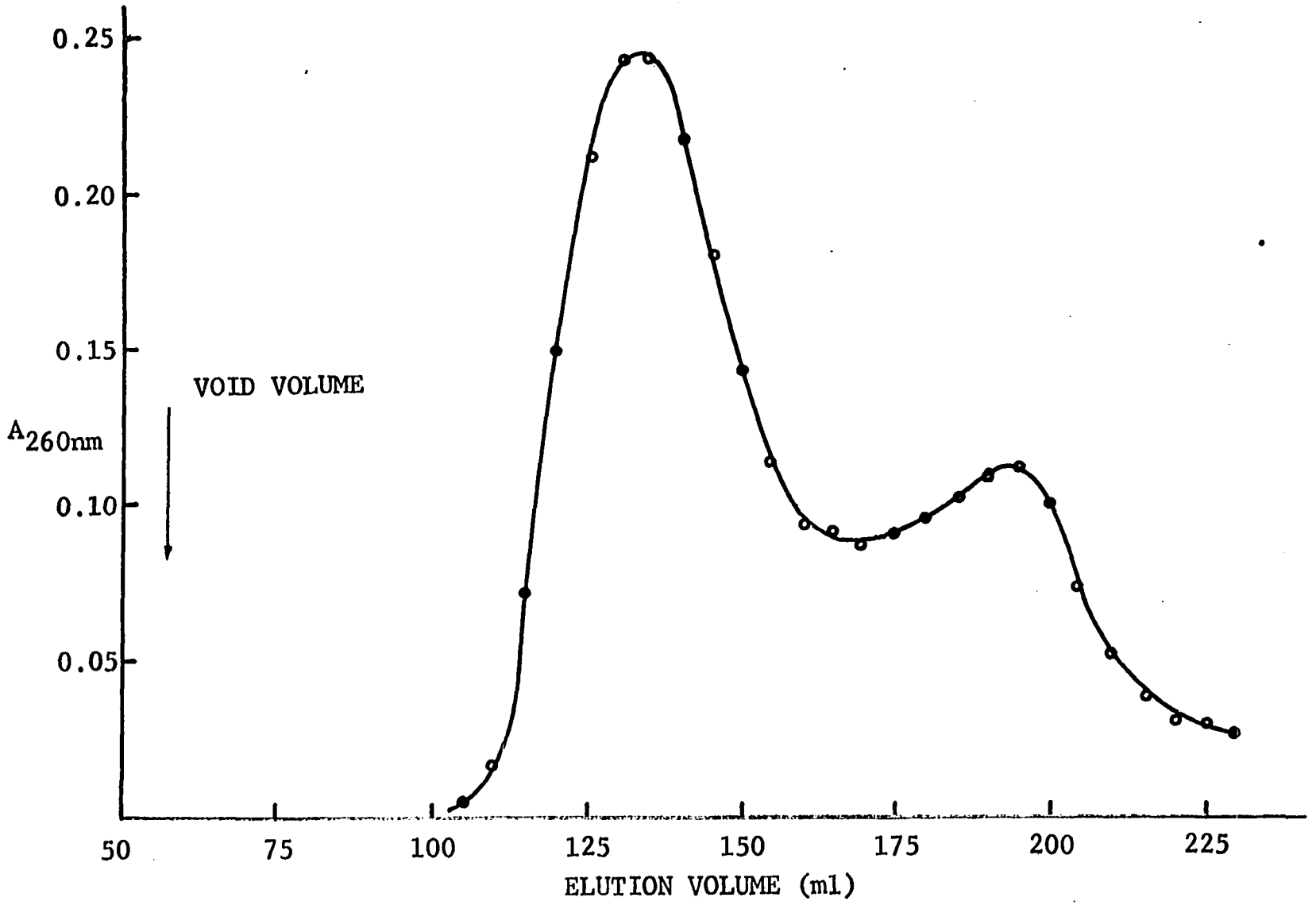

0.50
0.35
0.30
A_{280nm}
0.10
0.05
GEL
PRECIPITIN ACTIVITY
50
75
100
125
150
175
200
225

commercially. However, a preparation of 80s porcine liver ribosomes were obtained through the generosity of Dr. Marshall Phillips of the National Animal Disease Laboratory. This ribosome preparation contained primarily 80s particles (90%) when examined in the Model E ultracentrifuge.

A portion of this preparation was applied to the same Sepharose 2B column used for the fractionation of the P. multocida 40-P preparations. The eluant, flow rate and the size of the collected fractions were the same as those used in the above experiments. The results of the gel filtration of these reference 80s ribosomes are shown in Figure 9. As can be observed in this elution profile the major component eluted had a maximum 260 nm elution volume of approximately 132.5 ml. The elution curve was slightly more symmetrical and not quite as broad as the elution curve obtained for the major (S-II) component of the P. multocida preparations. The smaller peak appeared near the total internal volume of the column indicating it was considerably lower in molecular weight. When the ultraviolet spectra of the two peaks were compared, the larger peak had an ultraviolet spectra characteristic of mammalian ribosomes while the smaller peak's spectra resembled that of ribonucleic acids.

Although there are a very small number of published reports on the gel filtration of yeast and bacterial ribosomes on agarose, it appears that this is the first

Figure 9. Sepharose 2B gel filtration of a formalin-fixed preparation of 80s porcine liver ribosomes on the same column and using the same conditions as previous experiments with the P. multocida P-1059 40-P preparations


0.25
0.20
0.15
0.10
0.05
A_{260nm}
VOID VOLUME
50
75
100
125
150
175
200
225
ELUTION VOLUME (ml)

report of the gel filtration of mammalian 80s ribosomes on Sepharose 2B. Because of this it is not possible to compare the results obtained here with those in the literature. However, the results obtained with the ribosomes are consistent with the relative elution volume expected for particles of their reported size. A further discussion of these results can be found in a following section of this dissertation devoted to the calculation of Stokes radius and molecular weights of both the P. multocida S-II complex and the 80s ribosomes.

The Results of Biological Studies of the Gel Filtration Fractions

Serological reactivity

Each fraction from the Sepharose 2B gel filtration column was tested for its ability to react with rabbit antisera to the crude 40-P preparation. This was done by the Ouchterlony gel-diffusion method as described in Methods. The fractions representing the peak I substance did not react with the antiserum in gel diffusion plates. The fractions designated peak II or S-II, however, gave an intense precipitin line in Ouchterlony plates. Only one precipitin line was observed in the fractions as they were collected from the column. However, after the fractions representing S-II were pooled and concentrated, two lines were observed close to the antigen well. An example of this reaction is included in Plate 1a. Two

precipitin lines were also observed in concentrated pooled tubes representing the leading, trailing, and center portions of S-II. The fractions from peak III gave one to three precipitin lines when significant amounts of the peak III component was observed in a preparation. These precipitin lines did not form lines of identity or spurs with the precipitin lines observed in concentrated peak S-II fractions indicating they were not serologically related. The precipitin lines from peak S-III fractions were located close to the antiserum well and formed continuous lines with the faster diffusing precipitins of the original 40-P preparations.

Immunogenic properties

The ability of fractions from Sepharose 2B columns to actively immunize 16-18 gm female mice was determined. The immunogenicity of fractions from the Sepharose 2B column chromatograph of a Diaflo X-M 100 concentrate of a whole culture filtrate was tested first. The actual elution profile of this concentrate is shown in Figure 8. The fractions representing the smaller peak (elution volumes of 85-145 ml) were pooled and designated the S-II substance, while the fractions representing the larger peak were pooled (elution volumes of 170-210 ml) and referred to as the S-III substance. The results of these immunity studies are shown in Table 14. Each mouse was inoculated i.p. once and then challenged 5 weeks later with 1040 *P. multocida* P-1059 virulent organisms (determined by

Table 14. The active immunity of mice induced by inoculation with Sepharose 2B fractions of a concentrated culture filtrate of P. multocida P-1059 Gray

Immunogen[a]	Dose µg	Dead at day no. 1	Dead at day no. 2	No. survivors / No. challenged	% Protection
Component S-II	18	2	4	4/10	40
Component S-III	24	8	0	1/9	11
	48	9	0	1/10	10
The unfractionated culture filtrate concentrate	50	6	1	2/9	22
Unfractionated 40-P preparation	11	0	4	6/10	60
Controls	--	10	0	0/10	0

[a]Each mouse inoculated intraperitoneally once with sample in 0.1 ml saline and challenged 5 weeks later with 1040 P. multocida P-1059 F1 organisms. All mice were observed for 2 weeks following challenge and all mice which died during this period died within 2 days of challenge.

actual plate count). The results show that the S-II substance was more effective in protecting mice (40% protection) than the S-III substance (11%) and also resulted in a higher level of protection than the original Diaflo X-M 100 concentrate (22%). The highest level of protection (60%) resulted from an inoculation of a 40-P preparation.

A second immunization experiment was performed with the Sepharose 2B fractions of a 40-P preparation. A chromato-

graph of this preparation is shown in Figure 5. The fractions representing the first peak (elution volumes 50-70 ml) were pooled and designated S-II. Since this preparation contained only a small amount of material eluted near the total bed volume (i.e., S-III) it could not be analyzed for immunogenicity; however, fractions from the gel filtration of another 40-P preparation (see Figure 4) were pooled and designated component S-III (elution volumes 180-210 ml). The results of these immunity studies are shown in Table 15, where two equal dose inoculations were given 26 days apart and the 16-18 gm female mice were challenged with 980 organisms 3 weeks after the second inoculation. In this experiment the highest level of protection (100%) was provided by two inoculations with 24 µg of S-II. Fairly high levels of protection (83%) were also obtained with the peak S-I substance and the original unfractionated 40-P preparation. However, the peak S-III substance gave lower levels of protection (42%).

These immunity studies were carried out using mice rather than turkey poults as poults were not available at the time of experimentation. Young chickens were not used as they are resistant to P. multocida strain P-1059 infection (Heddleston, 1962).

Toxicity studies on the gel filtration fractions

The toxicity of the substances in pooled tubes representing peak I and peak II were determined by their inoculation on the

Table 15. The active immunity of mice induced by inoculation with Sepharose 2B fractions of a 40-P preparation isolated from P. multocida strain P-1059 Gray

Immunogen[a]	Dose µg	Dead at day no. 1	2	4	11	No. survivors / No. challenged	% Protection
Component S-I	10,10	0	1	1	0	10/12	83
Component S-II	24,24	0	0	0	0	12/12	100
Component S-III	50,50	3	4	0	0	5/12	42
Original 40-P preparation	20,20	0	1	0	1	10/12	83
Control	-- --	10	1	0	0	0/11	0

[a]Each mouse inoculated intraperitoneally with 2 equal doses of sample in 0.1 ml saline 26 days apart and challenged 3 weeks after second inoculation with 980 P. multocida P-1059 F1. Controls were not injected. Mice were observed for two weeks following challenge.

chorioallantoic membrane of 10 day old chick embryos. Because of the limited amounts of peak I and peak III, it was not possible to test the toxicity of peak III and peak I was only tested in one concentration. The peak II substance was tested at five different concentrations and 50% lethal dose (LD_{50}) was determined by the probit method. Peak I and peak II were from a Sepharose 2B fractionation of 40-P preparation lot 116 and a chromatograph of this preparation is shown in Figure 7 of the previous section on gel filtration. The results of the embryo toxicity studies are shown in Table 16. The LD_{50} of the peak

Table 16. Toxicity studies in chick embryos of Sepharose 2B fractions of a 40-P preparation from P. multocida P-1059 Gray[a]

Sample	Amount µg	Dead at no. hours 12	36	Total no. dead / No. challenged	% Dead
Peak I	38	3	1	4/12	33
Peak II	22.2	10	0	10/12	83
	11.1	9	0	9/12	75
	8.5	8	0	8/12	67
	5.5	3	4	7/12	58
	2.8	2	1	3/12	30
Unfractioned 40-P preparation wt 116	10.1	6	0	6/10	60
Control	diluent (formalinized saline)	0	0	0/10	0

LD_{50} (peak II) equals 5.2 µg (2.0 µg , 8.0 µg)[b]

[a]0.1 ml samples inoculated onto the chorioallantoic membrane of ten day old embryos and eggs candled for 5 days following inoculation.

[b]Values represent lower and upper 95% confidence limits of the LD_{50} value as determined by the probit method.

II substance was 5.2 µg with lower and upper 95% confidence limits of 2.0 µg and 8.1 µg respectively. In an earlier experiment on the original 40-P preparation as shown previously in Table 6, the LD_{50} was 8.7 µg with lower and upper 95%

confidence limits of 5.8 µg and 14.0 µg.

Particle Size Determination of the Gel Filtration Fraction S-II

Since the gel filtration of the 40-P preparations consistently resulted in the large almost symmetrical peak S-II, it was decided to use this data to estimate the particle size using gel filtration data and the method described by Ackers (1964) for determining the Stokes radius. This method can be used in combination with sedimentation velocity experiments to estimate particle weight. Electron micrographs were also taken of the column fraction S-II in order to use them as independent methods of estimating particle size.

Determination of the Stokes radius

The Stokes radius a of a particle can be estimated from gel filtration experiments by the method of Ackers (1964). This method depends on the theory developed by Ackers that the distribution coefficient (K_d) of a macromolecule on a porous gel medium can be equated to the Renkin equation describing a restricted diffusion process. The equation as written by Ackers is as follows:

$$K_d = (1 - \frac{a}{r})^2 \ (1 - 2.104 \ (\frac{a}{r}) + 2.09 \ (\frac{a}{r})^3 - 0.95 \ (\frac{a}{r})^5)$$

where K_d = the distribution coefficient

a = the Stokes radius of chromatographed substance

r = the effective pore radius of the gel medium.

Since the determination of the distribution coefficient (K_d) is required for the determination of the Stokes radii, the values of K_d were calculated from the peak effluent volumes of the major component (peak II) of the 40-P preparations from P. multocida P-1059. In addition the K_d values for the major components of a preparation of 80s porcine liver ribosomes and for a solution of Blue Dextran were determined. The values of K_d as well as values for another commonly used distribution coefficient (K_{av}) were calculated according to the following equations and summarized in Table 17.

The value K_d was calculated using the equation of Gelotte (1960)

$$K_d = \frac{V_e - V_o}{V_i}$$

and K_{av} was calculated from the equation of Laurent and Killander (1964):

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_o = the void volume determined by the elution volume of a high molecular weight component of Blue Dextran.

V_i = the internal or imbibed volume determined by the difference in the elution volume of a low molecular

weight compound (CH_2O) and the void volume.

V_t = the total bed volume determined by direct measurement of the column volume.

V_e = the elution volume determined by the volume of eluant collected from the time of sample application to the midpoint of the concentration peak.

The values for the various volumes determined for a Sepharose 2B and Sepharose 4B column are shown in the following table.

Table 17. Characteristic elution volumes of the Sepharose columns

Volume	Sepharose 2 B	Sepharose 4B
V_o	57.5 ml	45 ml
V_i	137	129
V_t	201	-
V_e (*P. multocida* S-II)	120	75
V_e (Ribosomes)	132.5	-
V_e (Blue Dextran)	145	-

The equations for K_d and K_{av} along with the above values were used to calculate the distribution coefficients shown in Table 18.

Table 18. Distribution coefficients on Sepharose 2B

Sample	K_d	K_{av}
P. multocida (Peak II)[a]	0.454	0.435
Swine ribosomes (80s)	0.545	0.523
Blue Dextran	0.636	0.610

[a]The K_d value of the P. multocida main component was 0.233 on Sepharose 4B.

The Ackers (1964) publication includes an extensive table of the values of a/r for a given distribution coefficient (K_d). Through the use of the determined K_d and the appropriate ratio a/r, the Stokes radius of an eluted macromolecule or particle can be determined if the gel pore radius (r) is known.

Since suitable materials of known Stokes radius were not available in order to determine the effective gel pore radius (r) according to the method of Ackers, it was necessary to use the values found in the literature. The r value used for the Sepharose 2B was 108.1 nm and is the value reported by Ackers (1964) for a 2% agar gel. The value used for the Sepharose 4B was 67 nm, which has recently been reported by Page and Godin (1970).

Using these values the Stokes radius of the major component of 40-P sediments, Sepharose 2B fraction S-II, was found to be 18.2 nm. The Stokes radius was determined to be 18.9 nm from the distribution coefficient calculated from Sepharose 4B column fractionations.

The Stokes radius was also determined for a preparation of 80s porcine liver ribosomes. The results of the gel filtration of the ribosomes are described in the preceding section of this dissertation. The Stokes radius of the ribosomes determined by Sepharose 2B gel filtration was 14.4 nm.

The values for K_d, the respective a/r and r literature values and the resulting Stokes radius are shown in Table 19.

Table 19. The Stokes radius of the major immunogenic fraction of P. multocida P-1059 and of a preparation of 80s ribosomes

Material	K_d	a/r[a]	r	Stokes radius
P. multocida				
Sepharose 2B	0.454	0.1682	108.1 nm[b]	18.2 nm
P. multocida				
Sepharose 4B	0.233	0.2822	67 nm[c]	18.9
Porcine ribosomes				
Sepharose 2B	0.545	0.1333	108.1 nm	14.4

[a]Values calculated from table of Ackers (1964).

[b]Value determined by Ackers (1964).

[c]Value determined by Page and Godin (1970).

Electron micrographs of the Sepharose 2B fraction S-II

Electron micrographs were taken (courtesy of Mr. Alfred E. Ritchie, National Animal Disease Laboratory, Ames, Iowa) of fractions obtained from Sepharose 2B gel filtration of the P. multocida P-1059 preparations. The electron micrographs of the main fraction (peak S-II) from the Sepharose 2B column are shown in Plate 3b. A large number of spherical particles are evident in Plate 3b,A where the greatest percentage of the particles had diameters of 34-39 nm. By measuring the diameters of a large number of particles the average diameter was found to be 37 nm. Plate 3b,A (113,190 x) also shows a number of smaller particles with diameters of approximately 14-20 nm and a few particles as large as 70-80 nm. A number of small cylindrical or rod-shaped particles were also observed and generally had dimensions of 9 x 20 nm.

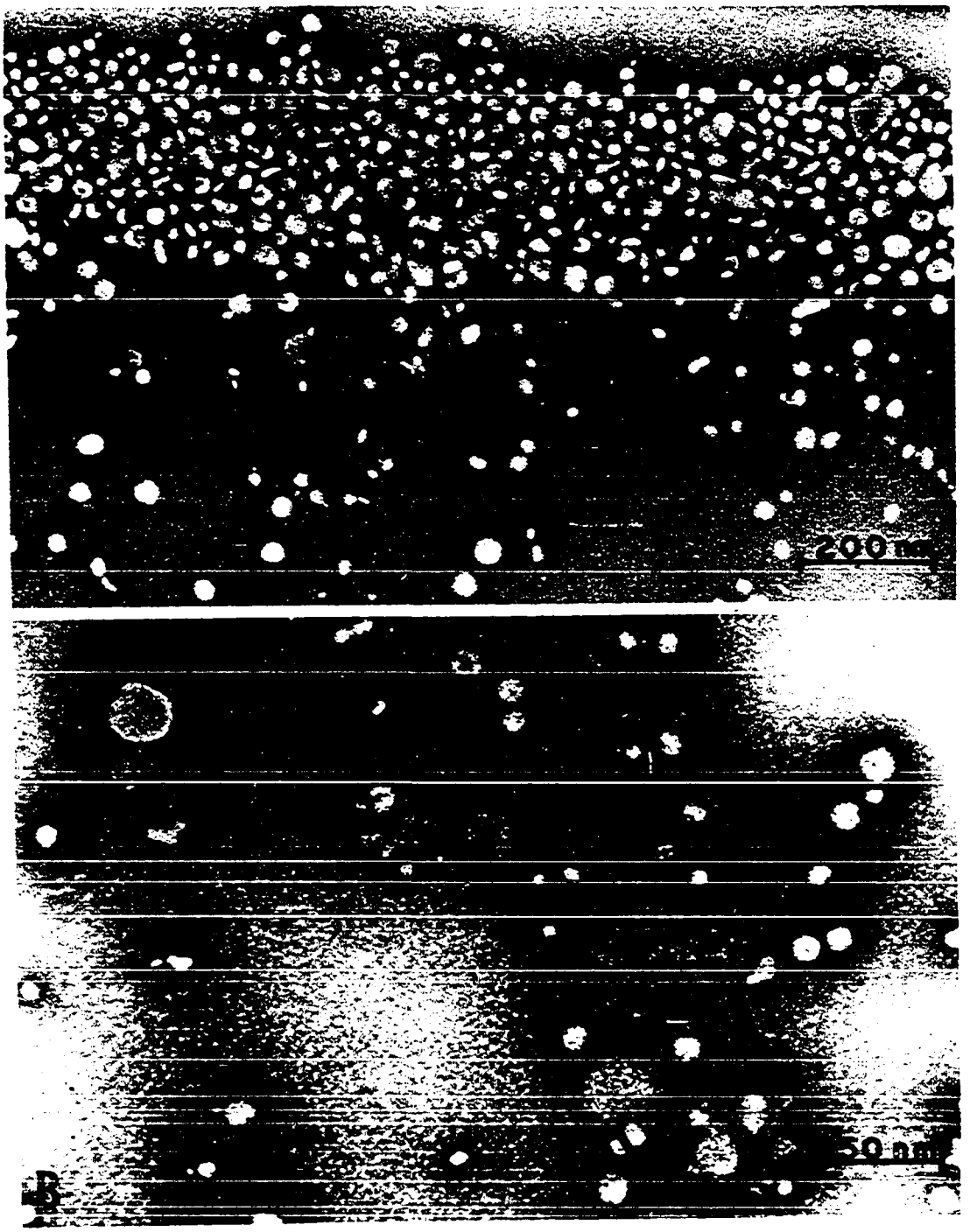
Plate 3b,B is a higher magnification (144,900 x) of the same preparation. In the upper left hand corner is one of the larger particles which has an approximate diameter of 75 nm. All preparations were in a potassium phosphotungstate negative stain.

The results of these electron micrographs are consistent with the gel filtration determinations of the Stokes radius. For if the particles are spherical as indicated by the electron micrographs, the Stokes radius of 18.2 - 18.9 nm would be the radius of spheres having the diameters of 36.4 - 37.8 nm.

Plate 3b. Electron micrographs of the Sepharose 2B peak S-II immunogenic and toxic complex; preparations in phosphotungstate negative stain

A. Top photograph has magnification factor of 113,190 x.

B. Bottom photograph has magnification factor of 144,900x.


200nm
B
50nm

Similar spherical particles have been observed in crude ultracentrifuged (105,000 x g) preparations isolated from Pasteurella multocida (X-73 gray mutant) as reported by Heddleston et al. (1966). In this investigation electron micrographs were also taken of shadow casted preparations and the particles or vesicles were found to be flattened. The X-73 particles were described as being membranous vesicles and the P. multocida P-1059 particles appear very similar.

Estimation of the Particle Weight of the Sepharose 2B Fraction S-II

The particle weight of the immunogenic fraction S-II from the Sepharose 2B column was estimated by a procedure described by Ackers and Steere (1967). This procedure makes use of the data obtained from sedimentation velocity experiments and the diffusion coefficient as determined from the Stokes radius.

The results of sedimentation velocity experiments

Sedimentation velocity experiments were performed using crude 40-P immunogenic preparations and the more purified Sepharose 2B fraction S-II. The crude 40-P preparations were found to have more variable sedimentation rates than the Sepharose 2B fraction. The sedimentation rates varied from 94 to 118 Svedberg units for the crude preparations while the S-II fraction had sedimentation rates from 98 to 102 Svedberg units.

The Schlieren sedimentation patterns of the two types of preparations differed in that the crude 40-P preparation showed an accumulation of material at the bottom of the cell soon after the rotor reached its maximum speed of 29,500 rpm. A small amount of apparently lower molecular weight material was visible at the meniscus. These components were not seen in the S-II fractions which appeared to have one large peak (see Plate 4). In both types of preparations the main sedimentation peak broadened rapidly.

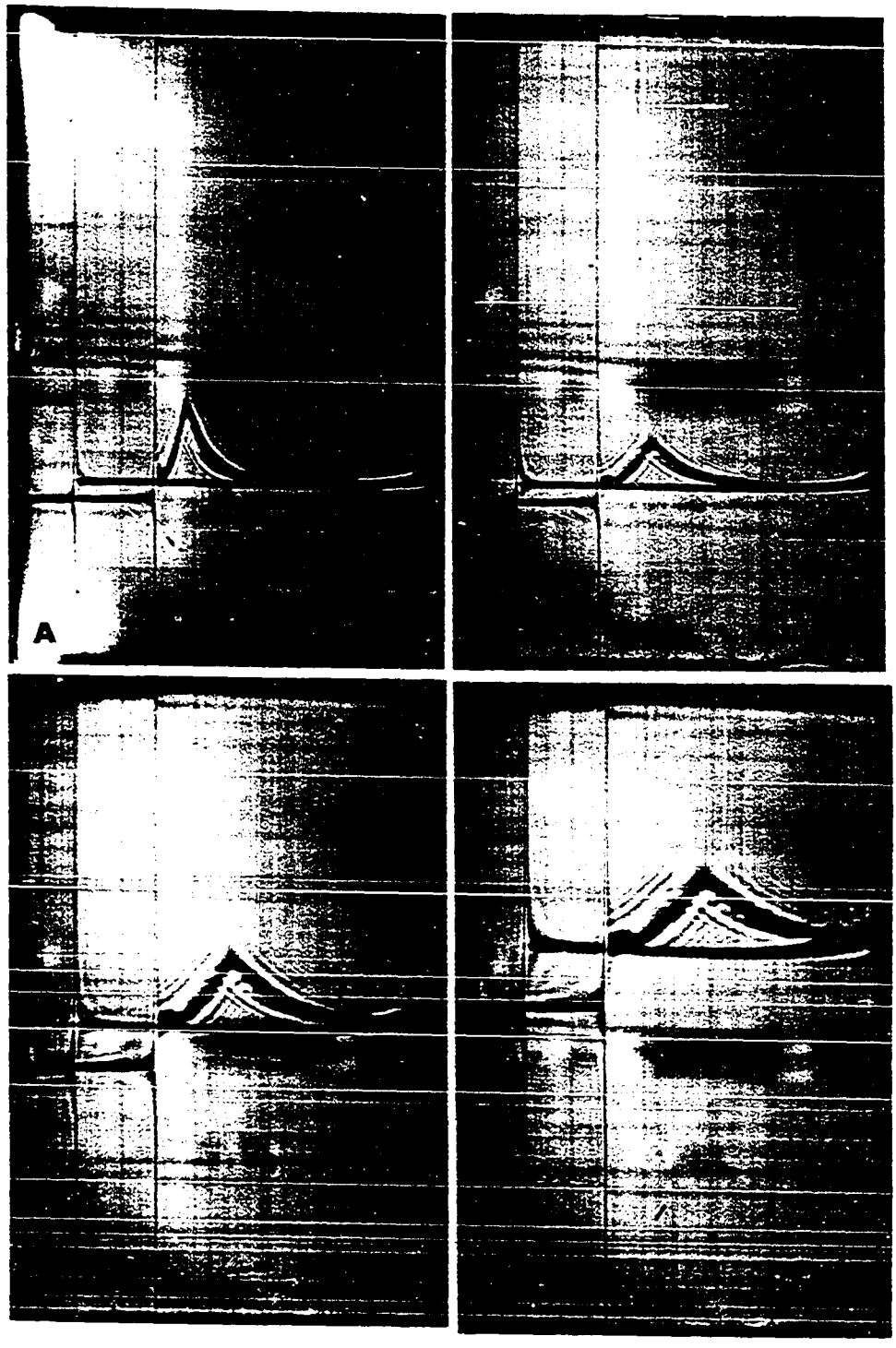
The observations reported above were for experiments performed at ambient temperatures and the solvent was 0.15 M NaCl. The sedimentation coefficients were corrected to a value corresponding to sedimentation in water at 20°C.

Estimate of diffusion coefficients The diffusion coefficient was calculated for the P. multocida P-1059 S-II fraction from the Stokes radius using a procedure described by Ackers and Steere (1967). This method uses the Einstein relationship which is given in the methods section of this dissertation. The estimated value for the diffusion coefficient for the Sepharose 2B S-II fraction was 1.19×10^{-7} cm^2/sec.

The diffusion coefficient was also calculated for the major Sepharose 2B fraction of the formalinized 80s ribosome preparation. The diffusion coefficient was 1.45×10^{-7} cm^2/sec. for the ribosomes. Values of $1.3 - 1.4 \times 10^{-7}$ cm^2/sec have previously been reported for the diffusion coefficient of 80s ribosomes (Spirin and Gavrilova, 1969).

Plate 4. Sedimentation velocity patterns of the P. multocida immunogenic complex after purification by Sepharose 2B gel filtration

The Sepharose 2B fraction S-II concentration was approximately 9 mg/ml, in 0.15 M NaCl containing 0.1% w/v formalin. The rotor speed was 29,500 rpm and runs were made at ambient temperature. Frame A was taken after the rotor reached speed and frames B, C, and D at 2 minute intervals.


A

Estimation of particle weight

Molecular or particle weights can be determined by the combination of the values for the sedimentation coefficient and the diffusion coefficient of a particle through the use of the Svedberg equation:

$$M = \frac{RT\ S_{20,w}}{D_{20,w}(1 - \bar{V}\rho)} \quad .$$

In this equation R is the gas constant, T the absolute temperature, $S_{20,w}$ the corrected sedimentation coefficient, $D_{20,w}$ the diffusion coefficient, $\bar{V}$ the partial specific volume and ρ the density of water at $20^{o}C$.

This equation was used to calculate the particle weight of the P. multocida immunogenic substance eluted in peak II from the Sepharose 2B column using the previous mentioned values for the sedimentation coefficient and diffusion coefficient. Using the values of $S_{20,w} = 100$, $D_{20,w} = 1.19 \times 10^{-7}$ cm^2/sec and $\bar{V} = 0.74$ the calculated particle weight was 7.91×10^6 for the immunogenic complex.

The value of 0.74 cm^3/gm was used for the partial specific volume of the immunogenic complex. This value was used because the bouyant density of the immunogenic complex in potassium bromide gradients as described in the following section was slightly less than the bouyant density of bovine serum albumin. This would indicate that the immunogenic complex might have a slightly higher partial specific volume ($\bar{V}$) than bovine serum

albumin which reportedly (Phelps and Putnam, 1960) has a ($\bar{V}$) of 0.734 cm^3/gm.

The same procedure was used to estimate the particle weight for the formalinized 80s porcine liver ribosomes. The calculated particle weight was 4.06×10^6 when using values of $S_{20,w} = 80$, the estimated diffusion coefficient of 1.45×10^{-7} cm^2/sec and a value for ($\bar{V}$) of 0.66 cm^3/g as reported by Spirin and Gavrilova (1969). This estimated particle weight for the ribosomes is close to the values ($4.11 - 4.7 \times 10^6$) reported by other investigators for mammalian ribosomes (Spirin and Gavrilova, 1969).

The calculated physical properties of the immunogenic fraction S-II isolated from the P. multocida P-1059 Gray culture filtrates as well as the physical properties of the porcine liver ribosomes used as a reference macromolecular complex are summarized in Table 20.

Determination of the buoyant density

The buoyant density of the immunogenic S-II fraction isolated from P. multocida was determined in both potassium bromide (KBr) and sucrose equilibrium centrifugation. The best results were obtained with KBr density gradient centrifugations as stable linear gradients were formed in approximately 38-40 hrs. when centrifugations were performed at 40,000 rpm in a SW-65 Spinco rotor maintained at 25°C. It was necessary to centrifuge sucrose gradients for 60 hours or

Table 20. Physical properties of the immunogenic fraction S-II of P. multocida P-1059 Gray and of a reference preparation of porcine liver ribosomes

Properties	P. multocida fraction S-II	Porcine liver[a] ribosomes
Sepharose 2B distribution coefficient, K_d	0.454	0.545
Stokes radius, a	18.2 nm	14.4 nm
Diffusion coefficient $D_{20,w} \times 10^7$ cm^2/sec	1.19	1.45 (1.3-1.4)
Sedimentation coefficient $S_{20,w}$ Svedberg	100s	80s
Particle weight	7.91×10^6	4.06×10^6 ($4.1-4.7 \times 10^6$)
Dimensions in electron microscope (negative staining)	37 nm average diameter of spheres	(28-30 nm x 22 nm)

[a]Ribosome values in parenthesis represent literature data for mammalian ribosomes as tabulated by Spirin and Gavrilova, 1969.

longer at 40,000 rpm to ensure the stable banding of the light scattering zones resulting from the application of P. multocida P-1059 immunogenic preparations.

The determination of when equilibrium conditions existed was aided in a very significant manner through the use of buoyant density markers. The relative positions of the buoyant density markers and light scattering zones were

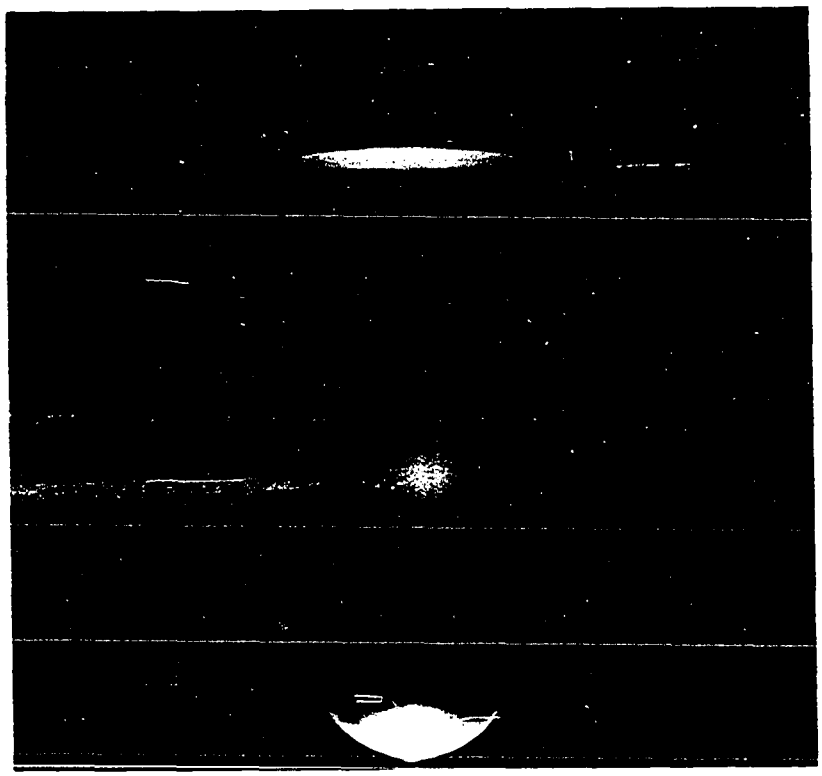
recorded at various time intervals. When there was no change in the relative positions for an 8 to 12 hour time period, the tubes were fractionated and the densities of the fractions determined through the use of a refractometer as described in Methods.

An example of the light scattering zones resulting after a KBr density gradient equilibrium centrifugation of the immunogenic 40-P preparation is shown in Plate 5. Although not immediately obvious in the photograph, there were at least two distinct regions of light scattering. The upper zone i.e. the one with the lowest density is the one most obvious in the photograph and scattered light more intensely. Immediately below this zone was another light scattering region which appeared to consist of two very narrow bands. Direct observation of KBr density gradients of the Sepharose 2B fraction S-II resulted in a similar observation, however, the lower i.e. more dense region was much less pronounced.

The ultraviolet absorption of each fraction at a wavelength of 280 nm was determined in a spectrophotometer. In order to reduce the absorption of KBr it was necessary to dilute each fraction with distilled water. A solution of KBr with the density of the most dense region of the gradient was diluted an equal amount and used as the blank. No ultraviolet absorbing peaks were observed in fractions from control tubes. The ultraviolet absorption pattern for the 40-P preparation

Plate 5. Light scattering zones observed in tubes after potassium bromide equilibrium density gradient centrifugation of P. multocida immunogenic complexes

Approximately 0.3 ml of a 40-P preparation containing 7.2 mg/ml, in 24% w/w KBr was added to the top of the tube containing 1.6 ml of each of 3 KBr solutions, 28% (w/w), 32% and 36%. Centrifuged in SW 65 rotor for 38 hrs. at 40,000 rpm and 25°C.



from P. multocida P-1059 is shown in Figure 10. The ultraviolet absorbing peak with a density of approximately 1.285 gm/cm^3 corresponded to the more pronounced light scattering zone and the peak with a density of 1.304 gm/cm^3 corresponded to the zone which scattered less light.

Each fraction was examined for its ability to react with antiserum in Ouchterlony gel diffusion plates. An example of the results is shown in Plate 6. The fraction with the most intense precipitin line (fraction #13 in this plate) had a density of 1.288 gm/cm^3. The fractions giving precipitin lines appeared to correspond to the less dense ultraviolet absorbing material and the more dense ultraviolet absorbing material did not appear to give a precipitin line. A summary of the P-1059 results is presented in Table 21.

A KBr buoyant density experiment was also performed with an immunogenic 40,000 precipitate from another avian strain of P. multocida, strain X-73 Gray. The results of this experiment are shown in Figure 11 where two ultraviolet absorption peaks are observed. Only the peak with the lower density (1.260 gm/cm^3) gave a precipitin line in Ouchterlony plates when tested with rabbit antisera to the X-73 Gray 40,000 precipitate.

A KBr density gradient experiment was also performed using a highly purified preparation of bovine serum albumin. The results of this experiment are shown in Figure 12. The

Table 21. Buoyant density values of the P. multocida P-1059 immunogenic complex in different KBr gradient centrifugations[a]

Conditions of centrifugation	Density range	g/ml	Buoyant density
43 hrs, 60,000 rpm	1.171-1.347	.032	1.283
43 hrs, 60,000 rpm	1.173-1.342	.032	1.284
38 hrs, 40,000 rpm	1.218-1.328	.025	1.288
60 hrs, 40,000 rpm	1.263-1.342	.015	1.286[b]

[a]All experiments performed at 25°C in a SW-65 rotor using an Spinco Model L-4 ultracentrifuge.

[b]Two u.v. peaks observed only with this gradient and only the lower density peak was serologically active.

main ultraviolet absorbing material had a buoyant density of 1.292 gm/cm^3 and this is very close to the published buoyant density value of 1.295 $\pm$ 0.005 gm/cm^3 reported by Cox and Schumaker (1961) for bovine serum albumin.

Buoyant density experiments were performed in sucrose density gradients using both P. multocida P-1059 Gray and X-73 Gray 40-P preparations. The experiments were performed in a SW-65 rotor at 60,000 rpm (258,000 x g), 9°C for 72 hours. The buoyant density of P. multocida P-1059 Gray and X-73 Gray 40-P preparations was 1.24 gm/cm^3 and 1.20 gm/cm^3 respectfully. The buoyant density values were calculated from the fraction which gave the most intense gel diffusion precipitin line.

Figure 10. Equilibrium centrifugation of an immunogenic 40-P preparation from P. multocida P-1059 Gray in a potassium bromide density gradient. Centrifugation at 40,000 rpm (114,000 x g), 25°C for 60 hours in an SW-65 rotor

Curved line through solid circles (o-o-o) represents the 280 nm u.v. absorption of fractions.

Line through open circles (o-o-o) represents the density of the fractions.

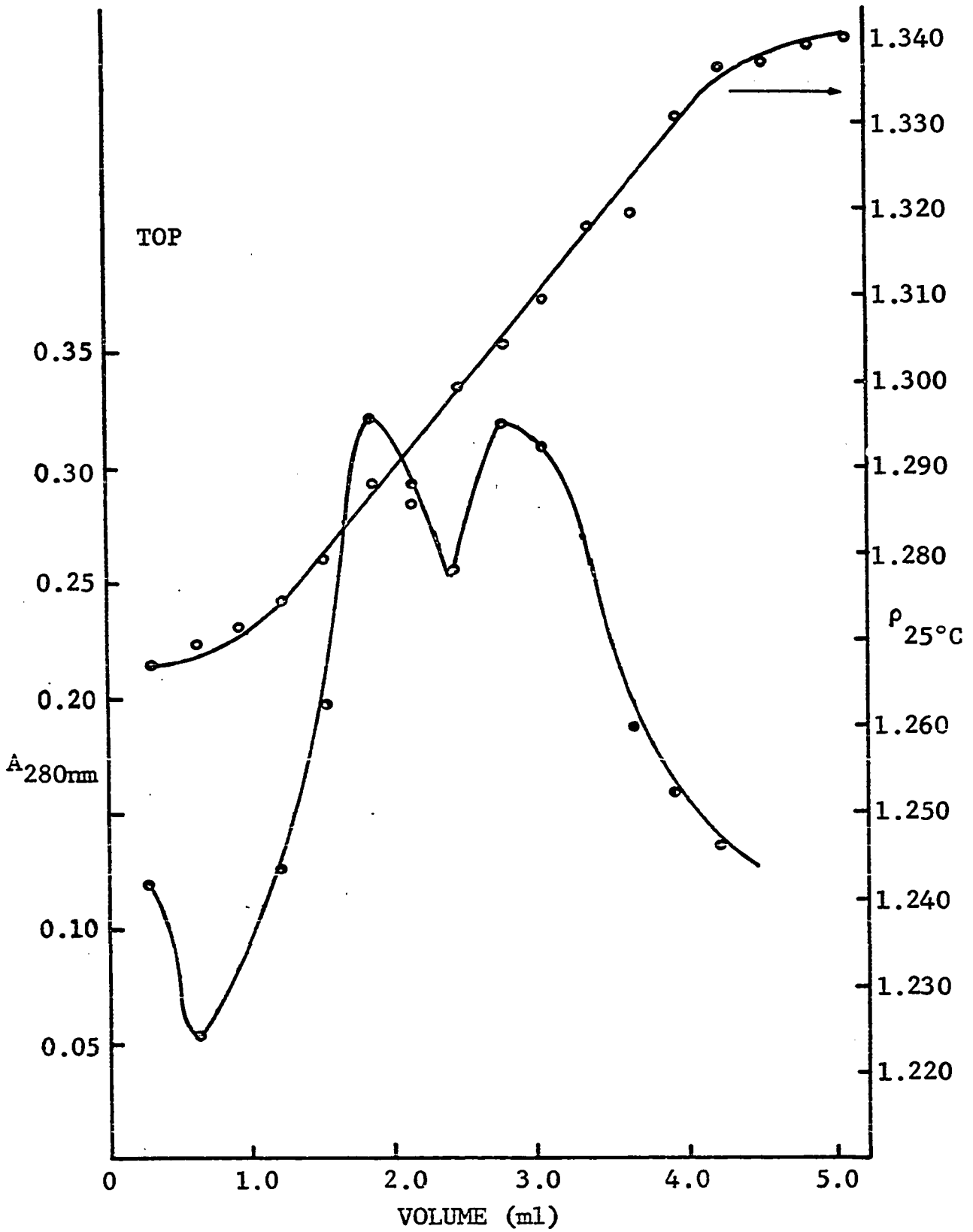

TOP
0.35
0.30
0.25
0.20
A_{280nm}
0.10
0.05
1.340
1.330
1.320
1.310
1.300
1.290
1.280
$\rho_{25°C}$
1.260
1.250
1.240
1.230
1.220
0
1.0
2.0
3.0
4.0
5.0
VOLUME (ml)

Plate 6. Immunodiffusion of the fractions obtained from a potassium bromide density gradient equilibrium centrifugation of an immunogenic complex from P. multocida

The numbered wells from 1-20 contain the twenty 0.25-0.30 ml fractions taken from the top with number 1 being the top fraction. The center row of wells contain a hyperimmune rabbit antisera (in 24% w/v KBr) to the immunogenic complex. Gradient and centrifugation conditions are the same as those of the previous plate. Density of fraction 1 was 1.218, fraction 13, 1.288 and fraction 20, 1.328.

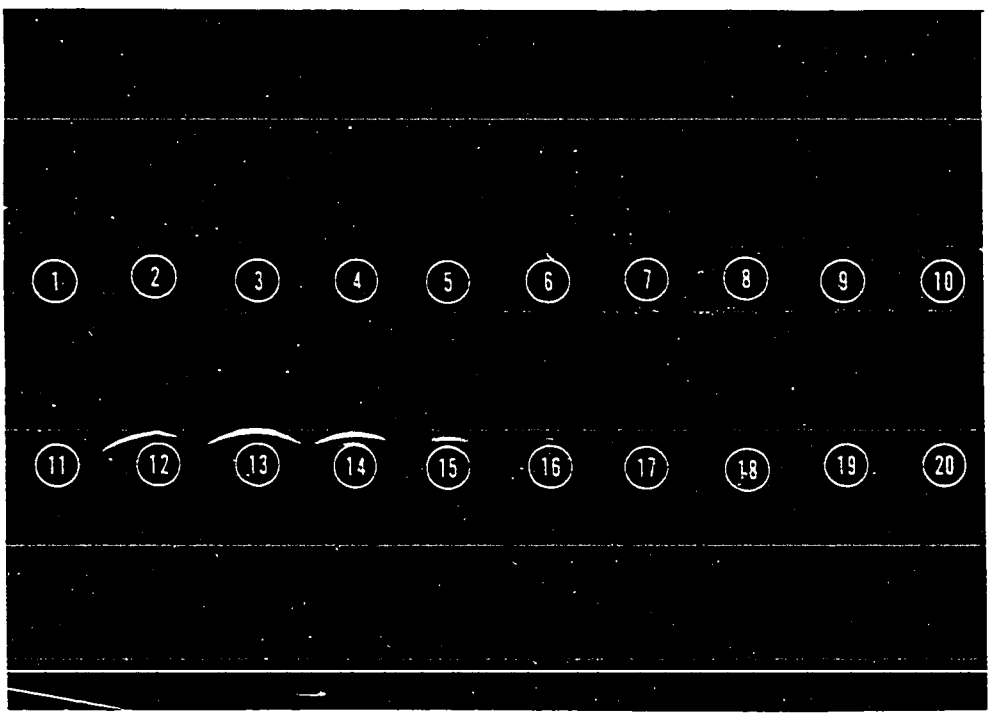

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

Figure 11. Equilibrium centrifugation of an immunogenic 40-P preparation from P. multocida X-73 Gray in a potassium bromide density gradient. Centrifugation at 40,000 rpm (114,000 x g), 25°C for 38 hours in an SW-65 rotor

Curved line through solid circles (o-o-o) represents 280 nm u.v. absorption of fractions.

Line through open circles (o-o-o) represents density of the fractions.

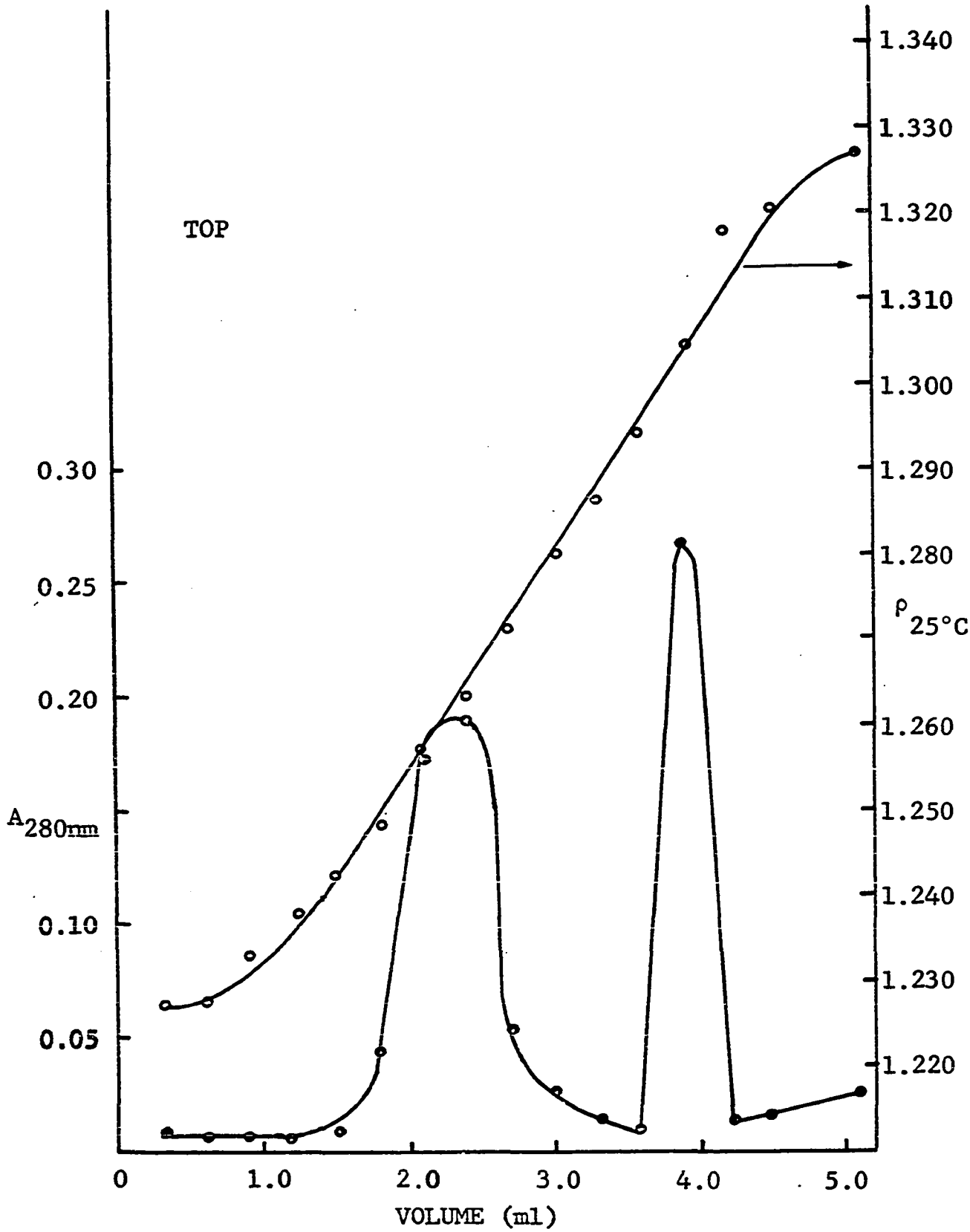
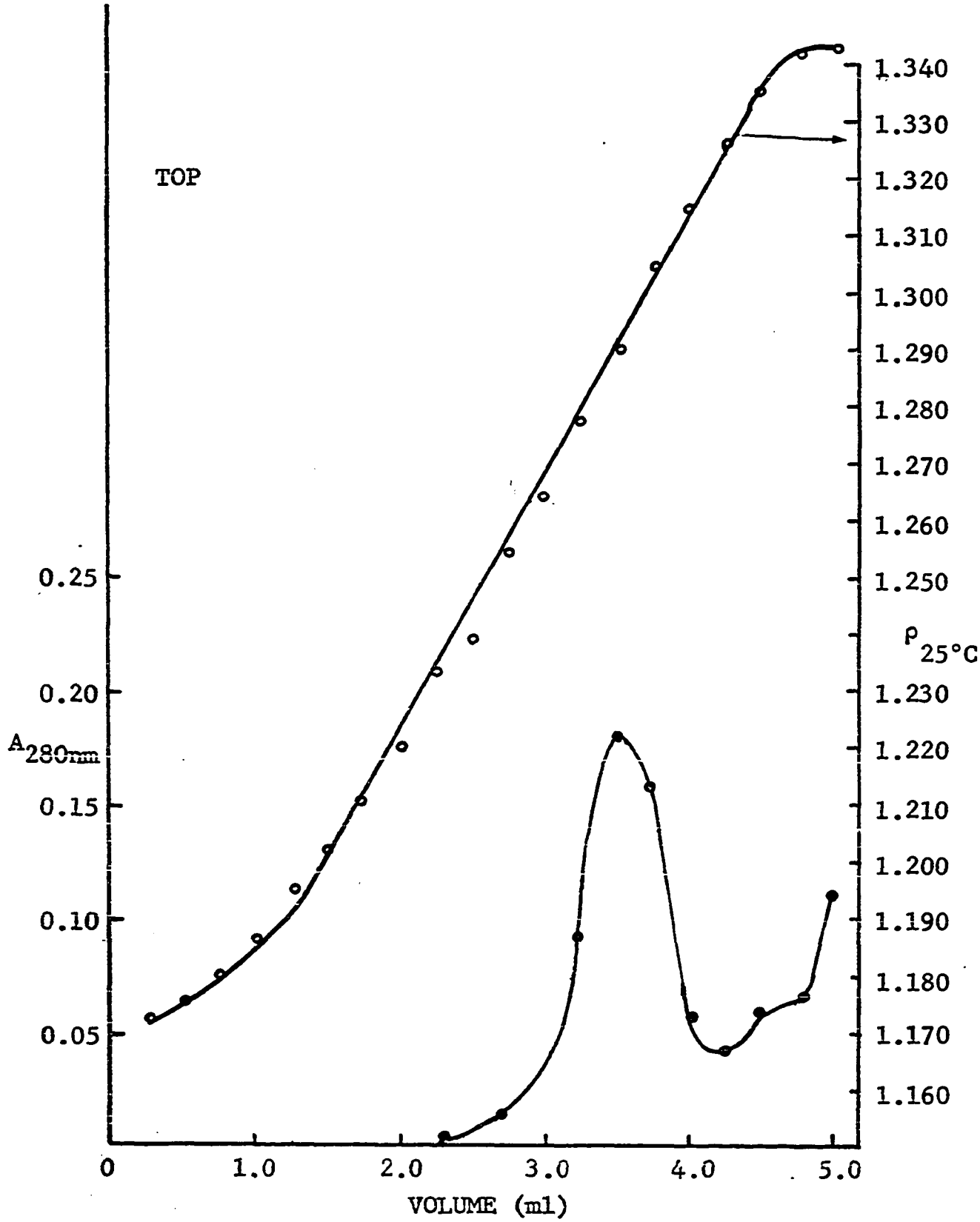

TOP
1.340
1.330
1.320
1.310
1.300
1.290
1.280
1.260
1.250
1.240
1.230
1.220
ρ 25°C
0.30
0.25
0.20
0.10
0.05
A 280nm
0
1.0
2.0
3.0
4.0
5.0
VOLUME (ml)

Figure 12. Equilibrium centrifugation of bovine serum albumin in a potassium bromide density gradient. Centrifugation at 60,000 rpm (258,000 x g), 25°C for 43 hours in an SW-65 rotor

Curved line through solid circles (o-o-o) represents the 280 nm u.v. absorption of the fractions.

Line through solid circles (o-o-o) represents the density of the fraction.


TOP
1.340
1.330
1.320
1.310
1.300
1.290
1.280
1.270
1.260
1.250
$\rho_{25°C}$
1.230
1.220
1.210
1.200
1.190
1.180
1.170
1.160
0.25
0.20
A_{280nm}
0.15
0.10
0.05
0
1.0
2.0
3.0
4.0
5.0
VOLUME (ml)

The results of the buoyant density experiments are summarized in Table 22. The KBr buoyant density value for the P. multocida P-1059 preparation is an average of four values ranging from 1.283 to 1.288 gm/cm^3. These values were obtained from three different shaped gradients prepared as described in Methods.

Table 22. Buoyant density values obtained by equilibrium density gradient centrifugation

Sample	Buoyant densities	
	KBr (g/cm^3)	Sucrose (g/cm^3)
X-73	1.260	1.20
P-1059	1.286 ± 0.005	1.24
Bovine serum albumin	1.292	
(Bovine serum albumin)	1.295 ± 0.005[a]	

[a]Published value of Cox and Schumaker (1961).

Electrophoretic Studies on the Sepharose 2B Fraction S-II

Electrophoresis experiments were performed using two different techniques to study the homogeniety and mobility of the immunogenic complex isolated from P. multocida. Both the Sepharose 2B fraction S-II and the original 40-P preparation were analyzed by composite agarose-acrylamide gel and cellulose

acetate electrophoresis.

Composite agarose-acrylamide gel electrophoresis

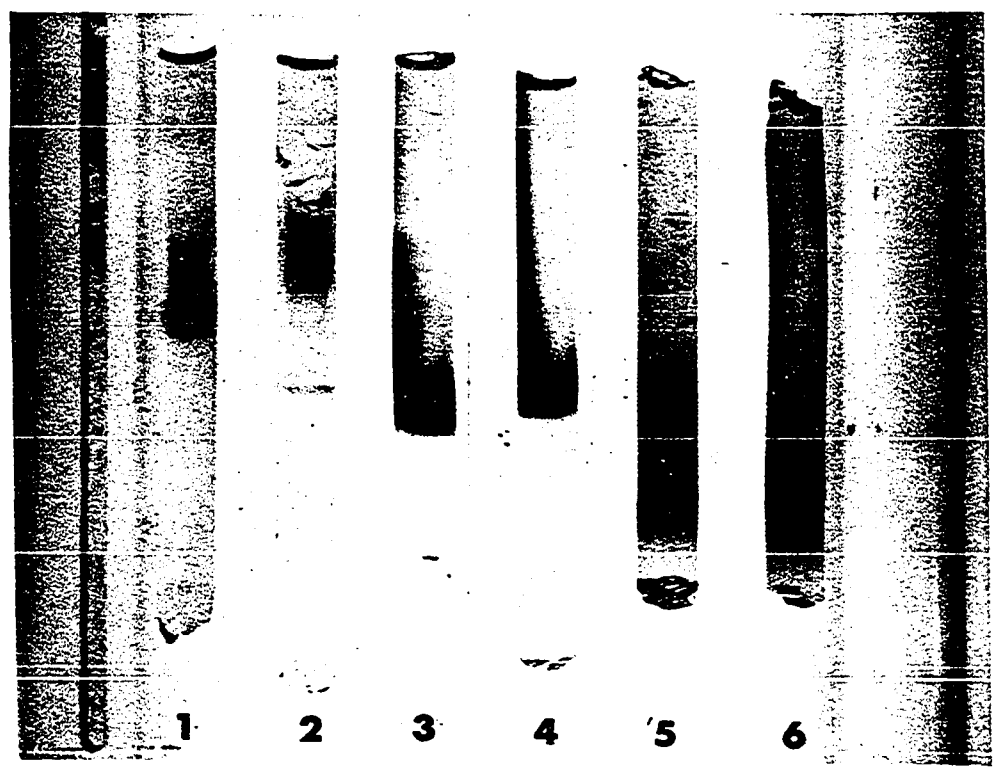
The established method of disc gel electrophoresis in acrylamide gels could not be used because the large immunogenic complex failed to enter 5% acrylamide gels and gels of a lower acrylamide content are extremely difficult to handle.

A composite gel containing 2.5% acrylamide and 0.8% agarose was prepared as described in Methods. The results of an electrophoresis experiment with this composite gel is shown in Plate 7. In the experiment shown in the plate, a Sepharose 2B fraction S-II, the original 40-P immunogenic preparation (lot 116) and commercial preparations of bovine serum albumin and ferritin were subjected to electrophoresis simultaneously. Electrophoresis was carried out at a constant current of 2.5 ma per gel, for 1-1/2 hours in .025 M Tris-glycine buffer pH 8.3. The immunogenic complex from the column (peak S-II) gave a single broad band when stained for proteins with Bromphenol Blue while the original 40-P complex gave a broad band plus a faster migrating narrow band. The ferritin and bovine serum albumin samples were run in duplicate to help determine the reproducibility of the migration. The gels containing the P. multocida preparations were also stained with the lipid stain, Sudan Black B, and a single intensely stained broad band was observed in the same position as the major protein band. An attempt was made to stain the gels for carbohydrate using

Plate 7. Composite agarose-acrylamide gel electrophoresis of the Sepharose 2B gel filtration peak II complex, the original 40-P preparation, and the proteins ferritin and bovine serum albumin

The sample applied to gel number 1 was the Sepharose 2B peak II complex; 2, the original 40-P preparation; 3 and 4, ferritin and 5 and 6, bovine serum albumin. Approximately 0.2 mg applied per gel.

Electrophoresis in .025 M Tris-glycine buffer, pH 8.3 for 1-1/2 hours at a constant current of 2.5 ma per gel. Gels stained with Bromphenol Blue.


1
2
3
4
5
6

a periodic acid-Schiff's reagent designed for regular acrylamide electrophoresis (Zacharius et al., 1969). When the gels were placed in the 12.5% trichloroacetic acid fixative, bands of percipitate were observed in the same position as the large protein and lipid band. However, after staining, it was impossible to destain the gel, even though agarose should be resistant to periodate. Consequently no band could be observed.

The bands shown in the photograph are rather broad in comparison to acrylamide gel electrophoresis bands which are sharpened by the Kohlrausch phenomenon in a discontinuous buffer system. This type of system was not used with the gels shown. However, some success was achieved in producing sharper protein and lipid bands by forming a sample gel of 1% w/v agarose on top of the composite gels and using a discontinuous buffer system. This method however suffered from the frequent formation of a concave junction between the sample and separating gel resulting in curved bands in the stained gels.

Cellulose acetate electrophoresis

Electrophoresis experiments were performed on samples of the immunogenic complex using a support medium of gelatinized cellulose acetate (strips of Cellogel, Colab, Co. Inc.). After electrophoresis for 1 hour, using 140 v, an initial current of 20 ma and a .05 M sodium borate buffer pH 9.0, the strips were stained for protein, carbohydrate and lipid.

The protein stain, Amido Black 10B, revealed two bands, a broad band with the higher mobility and a narrow band immediately behind it. The detection of carbohydrate with a periodic acid Schiff's reagent revealed a single broad band with the same mobility as the broad protein band. When the strips were stained with Fat Red 7B for lipid, a single band was observed with approximately the same mobility as the broad bands detected by carbohydrate and protein stains. Both the original 40-P preparations and the Sepharose 2B fraction S-II showed the same bands with no obvious differences.

DISCUSSION OF RESULTS

The initial results of this study have confirmed previous reports (Heddleston et al., 1966) that preparations which are immunogenic and toxic can be obtained from cultures of P. multocida P-1059 Gray. These preparations were obtained from saline solutions which were used to remove the bacterial cells from the surface of the agar solidified media. They were isolated by ultracentrifugation of the previously filtered solutions at 105,000 x g (40,000 rpm) for two hours. The sediments or precipitates were referred to as 40-P preparations. In addition to being toxic and immunogenic these preparations were found to be chemically and serologically complex.

The yield of these 40-P preparations isolated by a standard procedure from a given weight of cells was found to vary significantly from one bacterial lot to another. The exact reasons for this are not known but it was also observed that the actual yield of cells varied significantly from a given number of inoculated Roux bottles. This indicates that despite the use of a standardized procedure in growing and harvesting of the cells that the actual growth rate of the organisms was variable. Since the media used for growing the organisms is an undefined one, it is possible that slight variations in it may contribute significantly to the variation in the growth rate of the organism. Other investigators have shown that variations in the growing conditions of E. coli have

significantly altered the amount of a toxic lipopolysaccharide-protein complex which is excreted by the organisms (Rothfield and Pearlmen-Kotchenz, 1969). Variations in the concentration of divalent cations either in the medium or in solutions used for extraction have also affected the amount of a toxic complex recovered from E. coli (Rogers, 1971).

As mentioned above the 40-P preparations were found to be toxic and immunogenic. Since the primary purpose of this study was to characterize an immunogenic substance (i.e. a substance which specifically immunizes animals against infection with P. multocida), it was of interest to see if the toxic and immunogenic activities could be separated by fractionation.

Application of the 40-P preparations to columns of Sepharose 2B (2% agarose) followed by elution and collection of fractions separated the 40-P preparations into three characteristic ultraviolet absorbing fractions. These fractions or components of the 40-P preparations were designated peak S-I for the fraction eluted with the void volume, peak S-II for the major fraction, and peak S-III for the last peak eluted from the column.

The fractions were examined for their toxicity using chick embryos and mice. The most toxic fraction was the peak S-II substance. It appeared to be slightly more toxic in chick embryos (LD_{50} = 5.2 μg) than the original unfractionated 40-P preparation (LD_{50} = 8.9 μg). The peak S-I material was

slightly toxic but considerably less toxic than the S-II substance. The toxicity of the peak S-III substance was not determined due to a lack of sufficient material.

The ability of the three fractions to specifically immunize mice against lethal infections of P. multocida P-1059 was determined. The major peak S-II was found to be highly immunogenic in mice. Two injections of 24 µg which were spaced 26 days apart gave 100% protection to a group of 12 mice. It appeared to be slightly more effective than the original unfractionated 40-P preparation. The peak S-I material also protected mice. However, since the minimum effective dose of the peak S-II material was not determined, it was not possible to rule out the presence of a small amount of this immunogenic substance in the peak S-I material. The fraction designated peak S-III did not appear to be nearly as immunogenic as the S-II substance.

As a result of these studies the toxic and immunogenic activities of various preparations were found to be associated with the same major fraction S-II recovered from the Sepharose column. Analyses of the total recovery from the column using lypholiziation of dialyzed aliquots, microkjeldahl nitrogen determinations, as well as protein and carbohydrate analyses indicated that 87 to 95% of the applied samples were being recovered. Of the total recovered mass from the column approximately 80 to 90% was found in the major immunogenic and toxic fraction S-II.

Since there was a possibility that the preliminary process of ultracentrifugation of the culture filtrates produced artificial complexes, a gel filtration experiment was performed using a concentrated culture filtrate which had not been subjected to ultracentrifugation. Sepharose 2B gel filtration of this concentrate resulted in two fractions which corresponded to peaks S-II and S-III of the other experiments. The immunogenic activity was primarily in the S-II substance. This experiment demonstrated that the S-II substance was a naturally occurring material and not an aggregate or complex formed by the sedimentation process. It also demonstrated the effectiveness of the Sepharose 2B column for the S-II material was separated from a very large peak S-III which very likely contained a representation of all the other culture filtrate components.

Subsequent experiments were then carried out to test the relative homogeneity of the immunogenic S-II fraction as isolated from the column. Because of the rather obvious large size of the fraction as indicated by its distribution on the column, it was not possible to use many of the techniques used for studying the homogeneity of lower molecular weight macromolecules.

Physical, chemical, and serological techniques were used to examine the toxic and immunogenic preparations for their homogeneity. These techniques included sedimentation velocity experiments, electron micrographic studies, equilibrium density

gradient centrifugations and various forms of electrophoresis. In addition the fractions were examined in gel diffusion plates for the detection of precipitin lines brought about by the reaction with hyperimmune rabbit antiserum.

Electron micrographs taken of negatively stained preparations of fraction S-II indicated the preparations consisted of large numbers of almost spherical particles. Although there was a considerable range of different sized particles, a predominant number of the particles were found to have diameters of 35-40 nm. Earlier studies of rather similar toxic and immunogenic particles from another immunological type (X-73) of P. multocida had shown that these particles were flattened vesicles when studied by shadow casting (Heddleston et al., 1966).

A separate and independent method of estimating the particle size of the S-II fraction was also used. This technique uses the data obtained from the gel filtration columns and has been described by Ackers (1964). It relates the distribution coefficient obtained from the column and the Stokes radius of the eluted particles or maceomolecules. Using this method the calculated Stokes radius of the Sepharose 2B fraction S-II was 18.2 nm. A preparation of porcine 80s liver ribosomes was used as a reference sample since other suitable marker substances with known Stokes radii were not available for calibration of the column. Calculation of the

Stokes radius of this preparation from its Sepharose 2B distribution gave a value of 14.4 nm. This value is reasonable for the radii of 80s ribosomes which reportedly have dimensions of 28-30 x 22 nm and indicates the method is reliable.

If the S-II particles are assumed to be roughly spherical as they appear to be in the electron micrographs, then the Stokes radius of 18.2 nm is in good agreement with particles having diameters of 35-40 nm.

Sedimentation velocity experiments with the S-II fraction resulted in Schlieren patterns with only one peak having an approximate sedimentation coefficient of 100s. The unfractionated 40-P preparations also had a single peak but with these preparations there was an obvious accumulation of material at the bottom of the cell and material appeared to be present at the meniscus. These heavier and lighter molecular weight materials were apparently removed by the Sepharose 2B gel filtration.

The S-II fraction was also analyzed by carrying out equilibrium density gradient centrifugation in sucrose and in potassium bromide. Only one serologically active component was found in the fractions obtained from sucrose and potassium bromide gradients. The buoyant densities were 1.24 gm/cc and 1.286 gm/cc respectively. However, when the S-II fractions were analyzed with a potassium bromide gradient having a high resolving capability (i.e. a narrow gradient) two peaks were observed when the collected fractions were examined for their

ultraviolet absorption. Only the lighter component appeared to be serologically active and it was this component which showed the greatest amount of light scattering. It was not determined whether the other component which had a density of 1.304 was an impurity in the Sepharose 2B fraction S-II or whether it was a substance dissociated from the toxic and immunogenic complex by the action of the high concentration of potassium bromide. An inert peak with a density of 1.304 was also observed in the crude 40-F preparations only the ultraviolet peak was somewhat broader. This most likely indicates the presence of inert impurities presumably proteins.

Carbohydrates and lipopolysaccharides would be expected to pellet at the bottom of the potassium bromide gradients due to their relatively high buoyant density. Rothfield and Pearlmen Kotchenz (1969) reported that purified E. coli lipopolysaccharide had a buoyant density of 1.49 gm/cc. There was no obvious accumulation of material at the bottom of the gradients used for the analysis of the P. multocida preparations. However, the presence of a small amount of serologically inert material would not necessarily have been detected.

The immunogenic and toxic fraction from the column was also subjected to electrophoresis using two different kinds of matrix. Attempts to use disc electrophoresis with 5 and 7% acrylamide gels were unsuccessful as the S-II fraction failed to enter the gel matrix. For this reason, a composite gel

having a larger pore size was prepared. This composite gel was composed of agarose and acrylamide and the immunogenic and toxic fraction was found to migrate in this gel as a rather broad band. The broad band was detected with the same relative mobility when stained with either the protein strain Bromphenol Blue or the lipid stain, Sudan Black. This indicated that the S-II fraction was a complex of lipid and protein.

The S-II fraction was also subjected to electrophoresis on strips of a cellulose acetate (Cellogel and Sephaphore III). When the strips were stained with Amido Black 10B a protein stain, two protein bands were obtained. The band with the higher mobility was a rather broad band. When duplicate strips were stained for carbohydrate and lipid components, a band was observed in the smae relative position as this broad band. This indicated the S-II fraction contained a complex of protein, carbohydrate, and lipid. The second protein band observed with the S-II fractions was a narrow band which migrated immediately behind the broad band. This band may represent an "impurity" in the S-II fraction or it may represent a proteinaceous material which dissociated from an intact S-II complex during the electrophoretic process.

Isoelectric focusing experiments were performed using the 40-P preparations rather than the more "purified" Sepharose 2B fraction S-II. However, the particular 40-P preparation subjected to isoelectric focusing analysis was found to consist almost entirely of the S-II fraction when it was

applied to the gel filtration column. Thus the results with this preparation are presumed to be very similar to those which would have been achieved if the S-II fraction had been applied to the electrofocusing column. The sample migrated in the column to a position near the anode where the pH was in the range of 3.0 to 4.5. This band or zone was found to be distributed in an approximately symmetrical manner when analyzed by serological methods using Ouchterlony type gel diffusion plates. The fraction in the center of the zone had an pH of 3.7. No other precipitin lines were observed in the other areas of the pH 3-10 gradient. This indicated that the immunogenic fraction was serologically homogenous in terms of its isoelectric point.

Throughout the study of the P. multocida immunogenic complex Ouchterlony gel diffusion precipitin reactions have been used to study the antigenic complexity of various fractions. The initial sediments obtained by ultracentrifugation of the culture filtrates were found to give seven or more precipitin lines in gel diffusion plates using rabbit antiserum to the culture filtrates. However, the "washing" of these sediments by repetitive centrifugation was found to reduce the number of precipitin lines to two. These two precipitin lines were found close to the "antigen" well even when highly concentrated "antigen" preparations were used. This is an indication of the relatively high molecular or particle weight

of the antigenic materials. The slower diffusing precipitin component was only observed in highly concentrated preparations. After an extensive study and fractionation of the P. multocida immunogenic preparations it is felt that the slower diffusing substance is actually an aggregate of the main immunogenic complex, the faster diffusion component. Three separate pieces of evidence support this proposition: First, the leading, center and trailing portions of the major peak S-II eluted from the Sepharose 2B column gave a single precipitin line in gel diffusion plates but concentrated portions of these fractions gave two precipitin lines. It might be expected if two antigenic substances were present having different diffusion coefficients that they could be separated by the Sepharose 2B column. Second, the serologically active fractions obtained from isoelectric focusing experiments gave single precipitin lines which formed continuous lines when placed in adjacent wells indicating their serological identity. However, when the individual fractions were concentrated they each gave two precipitin lines. Thirdly, the serologically active fractions obtained from potassium bromide density gradients also gave single serologically identical precipitin lines. However, when the separate fractions were concentrated they also each gave two precipitin lines. Although it is frequently assumed when using these immuno-diffusion techniques that the presence of two precipitin lines indicates at least two separate

antigens, it appears in this case that the slower diffusing precipitin component may actually be an aggregate of the other component. The occurrence of aggregation in highly concentrated solutions is suggested by the fact that sediments are obtained from concentrates at lower centrifugal forces than from dilute solutions. It is, however, possible that the two bands of precipitate are caused by two different types of antibody. The various hyperimmune rabbit antisera used in these studies was not fractionated in order to separate the various classes of antibody.

In addition to the experiments carried out to examine the physical homogeneity of the toxic and immunogenic fraction, experiments were also performed to test its chemical homogeneity.

Since it was discovered that the carbohydrate distribution in the Sepharose 2B fractions was correlated with the ultraviolet absorption peaks, a tube by tube analysis was made in order to determine the ratio of protein to carbohydrate. The use of two protein determinations along with the carbohydrate determination indicated there was a relatively constant ratio of protein to carbohydrate in the S-II fraction. The slight variations in the ratios from one part of the eluted fraction to the other were felt to be within the experimental errors in the methods used. A re-cycling of a portion of the S-II fraction collected from one experiment, indicated that the ratios of protein to carbohydrate were approximately the

same after the second passage of the immunogenic complex through the columns. These facts indicate that the toxic and immunogenic fraction S-II is a complex containing a constant ratio of protein to carbohydrate within the limits of the analyses used.

Chemical analyses of the S-II fraction indicated that it contained 4.1% (w/w) nitrogen, 25-30% (w/w) protein and 10-11% (w/w) carbohydrate. The protein determinations were performed using both a differential ultraviolet absorption procedure and a modified Folin procedure. There was good agreement between the two. Bovine serum albumin was used as a standard. The phenol-sulfuric acid method was used for estimating the carbohydrate content with glucose as a standard.

Although the amino acid analysis was performed using an unfractionated 40-P preparation, subsequent analysis of this same preparation on the Sepharose 2B column indicated that it was more homogenous than most preparations. It did not appear to contain the primarily proteinaceous "impurity" S-III. For this reason the amino acid analysis of this preparation is fairly representative of the amino acids in the toxic and immunogenic fraction S-II.

Lipids were detected only in the S-II fraction so the fatty acid composition of the 40-P preparation presumably represents those found in the S-II fraction. The fatty acid composition of the preparation most likely represents both the fatty acids associated with the extractable phospholipid and

those associated with the Lipid A moiety of the lipopolysaccharide. Approximately 55% of the total fatty acid ester content was extractable with chloroform and methanol (2:1). This extract contained primarily phosphatidyl ethanolamine based on thin layer chromatograms. The discovery of phosphatidyl ethanolamine in the extracts was not surprising as biosynthesis studies of lipopolysaccharides have shown that phosphatidyl ethanolamine is an absolute requirement for the enzymes involved in the elongation of the polysaccharide side chains (Rothfield et al., 1966). The highest activity was obtained with a phosphatidyl ethanolamine containing two unsaturated fatty acid residues. The Lipid A moiety of lipopolysaccharide is really a mixture of all components liberated and precipitated upon short term acid hydrolysis of lipopolysaccharide. The Lipid A preparations generally contain saturated fatty acids (usually lauric, myristate, and palmitic) and β-OH fatty acids. These are generally attached to glucosamine moieties (Kasai, 1966).

Heptose analysis of the Sepharose 2B fractions indicated that an aldoheptose was predominantly located in the toxic and immunogenic fraction S-II therefore it is presumed that lipopolysaccharide a bacterial substance known to contain heptose is part of the complex. However, since these are no convenient methods of quantitating lipopolysaccharide, the actual percent of the complex which is lipopolysaccharide was not determined. However, the lipopolysaccharide content is

thought to be substantial as the P. multocida complex is about as toxic to mice and chick embryos as "protein free" lipopolysaccharides (Raynaud et al., 1964).

If one considers the buoyant density of the P. multocida complex (1.28 gm/cc) and the relative buoyant densities of protein (1.30 gm/cc), lipid (0.975 gm/cc), and lipopolysaccharide (1.49 gm/cc) (Rothfield and Pearlmen-Kotchenz, 1969), it is possible that the complex could contain from 40-50% w/w lipopolysaccharide with protein and phospholipid accounting for 25-30% w/w and 13-18% (w/w) respectively. A complex of this composition would have a theoretical buoyant density of 1.28-1.30 gm/cc.

Comparison of These Results with Other P. multocida Studies

Since there are very few complete chemical or physical studies of immunogenic macromolecules or complexes isolated from P. multocida, it is difficult to compare the immunogenic preparations described in this dissertation directly with the results found by others. However, various preparations studied by other investigators may have contained a similar immunogenic complex or components of the complex which were split off or dissociated from an intact complex.

The immunogenic complex described in this study may closely resemble the Boivin type toxic glycolipids described

by Pirosky (1938). However, extensive chemical or immunological studies were not reported for these glycolipids. A Boivin type antigen has also been reported by Prince and Smith (1966c) which was extracted from capsulated P. multocida. These authors found at least 16 soluble antigens which could be extracted from the organisms. Those most easily extracted from intact organism were presumed to be capsular substances and were designated α and β. The Boivin type antigen extracted by phenol was designated the γ antigen. The capsular α and β and the somatic γ were all felt to be type specific and important in immunological protection. Although no extensive studies of the chemical or physical nature of these antigens were described, the use of staining techniques indicated that possibly the α and β capsular antigens were complexes of protein and polysaccharide. The capsular component α was found to precipitate at a pH of 3.8 and was presumed to contain more protein than polysaccharide. Prince and Smith suggested that both capsular and somatic antigens were important for type specific protective vaccines.

The observation was made earlier by Knox and Bain (1960) that protein and lipopolysaccharide were precipitated by lowering the pH of saline extracts to 3.8. Inoculation with these isoelectric precipitates gave high levels of protection to mice when challenged with P. multocida. However, the lipopolysaccharide isolated by a phenol extraction procedure (Bain and Knox, 1961) from this bovine strain organism only

gave low levels of protection to inoculated mice. Perhaps the immunological protection of the initial isoelectric precipitates was caused by a complex of lipopolysaccharide and protein analogous to the complex of the avian strain of P. multocida whose isolation and characterization are described in this study. The isoelectric precipitation at pH 3.8 is very close to the value for the isoelectric point (3.7) found for the immunogenic complex described here.

Although Bain and Knox were not successful in providing mice with active immunity using the "purified" lipopolysaccharide, other investigators (Perreau and Petit, 1963), were able to passively protect mice with rabbit anti-lipopolysaccharide serum. This antiserum was prepared by injecting rabbits with a "purified" lipopolysaccharide absorbed to red blood cells. These studies are in agreement with the general observation that lipopolysaccharides are much more effective in producing antibodies in injected animals when they are coupled with protein. This fact is most likely the reason that the lipopolysaccharide complex studied in this investigation was so effective as an immunogen.

Other investigations of the immunogenic antigens of P. multocida type P-1059 have been reported recently by Brown et al. (1970) and Srivastava et al. (1970). The exact relationship of the immunogenic substances isolated by these investigators and the immunogenic complex described here are

not obvious. Srivastava et al. (1970) isolated their immunogenic antigen from a capsulated organism while the organism used throughout this study is a nonencapsulated form. The capsules of some strains of P. multocida have been shown to contain hyaluronic acid (Cifonelli et al., 1970). The use of a nonencapsulated strain in this study was deliberate in order to avoid the complicated mixtures which might occur if various capsular substances were present. Recent studies (Rebers et al., 1967) have shown that similar lipopolysaccharide complexes can, however, be isolated from encapsulated organisms.

There was no extensive effort in this work to attempt to study the nature of the forces which hold the components of the immunogenic complex together. Instead a basic effort was made to develop a method of isolation and purification of the intact complex which could be used routinely. However, the chelating agent EDTA which has been found recently by Rogers (1971) to dissociate the lipopolysaccharide-protein complexes of E. coli did not appear to affect the P. multocida immunogenic complex. P. multocida complexes which had been dialyzed in Tris-EDTA buffers and chromatographed on Sepharose 2B columns in the presence of Tris-EDTA buffer did not appear to have either a change in particle size or a change in their protein and carbohydrate content.

A recent report has advocated the use of Sepharose 2B for the purification of lipopolysaccharides (Romanowska, 1970). This investigator found that phenol extracted lipopolysaccharides were eluted from Sepharose 2B columns with the void volume. However, it is obvious from the studies reported on this P. multocida complex that lipopolysaccharide that is complexed with protein and phospholipid is not eluted with the void volume on Sepharose 2B in this case. Therefore, care must be taken in using this void volume elution position as a criteria of lipopolysaccharides for in many cases investigators may actually be working with lipopolysaccharide complexes rather than "purified" lipopolysaccharide.

SUMMARY

Reports of previous studies with P. multocida P-1059 Gray (Heddleston et al., 1966) have described the isolation of immunogenic and toxic sediments by ultracentrifugation of saline extracts at 105,000 x g for 2 hours. These sediments were reported to contain lipopolysaccharide and protein. The primary objectives of this study were: (1) to further purify the sediments obtained by ultracentrifugation, (2) to determine by fractionation procedures whether the immunogenic and toxic activities were associated with the same fractions and (3) to physically and chemically characterize the immunogenic substance.

The sediments or precipitates isolated from the saline "extracts" by ultracentrifugation were immunogenic for turkey poults with a single injection of 10 µg protecting 70% (7/10) of the challenged poults. The sediments were also lethal to chick embryos with a 50% lethal dose of 8.9 µg.

Chemical analysis of a number of different preparations demonstrated that they contained variable amounts nitrogen, phosphorus and carbohydrate. Amino acids, fatty acids and sugars were detected in hydrolyzates by gas chromatography. A full compliment of amino acids was found except for tryptophan which would be destroyed by the acid hydrolysis. A number of fatty acids were detected with lauric, myristic, and palmitic present in the highest amounts. The sugar

analysis indicated that galactose, glucose and D-glycero-L-mannoheptose were present based strictly on their retention time. This substantiated the sugar analysis previously carried out by thin layer chromatography and enzymatic assays. Extractable lipids were analyzed by thin layer chromatography and found to contain primarily phosphatidyl ethanolamine.

Since the sediments were not homogenous based on their variable chemical composition, various fractionation procedures were investigated in order to isolate a more "purified" immunogenic substance and to characterize it. Gel filtration experiments using Sepharose 2B (2% agarose) were successful in separating protein and carbohydrate "impurities" from a major fraction which was found to be as toxic and immunogenic as the original preparation. Gel filtration of a number of preparations indicated that this major fraction was characteristic of the preparations and had a reproducible distribution on the column.

Subsequent analyses were performed on this major fraction, designated peak S-II, in order to determine its homogeneity using physical, chemical and serological methods. The physical techniques used included equilibrium density gradient centrifugations, sedimentation velocity experiments, electrophoresis, isoelectric focusing and electron microscopic examination.

When individual tubes representing the major fraction S-II were analyzed for their protein and carbohydrate content, a relatively constant ratio of the two constituents was found, an indication of chemical homogeneity and the presence of a protein-carbohydrate complex. This immunogenic S-II fraction contained 4.1% (w/w) nitrogen, 25-30% (w/w) protein and 10-11% (w/w) carbohydrate. Cysteine-sulfuric acid reactions for carbohydrate indicated the heptose was located in the S-II fraction.

The results of the electrophoretic studies on composite agarose-acrylamide gels and cellulose acetate strips indicated the S-II fraction contained a complex of proteins, carbohydrate and lipid. An isoelectric point of 3.7 was determined on a preparation subjected to isoelectric focusing in a pH 3-10 gradient. Equilibrium density gradient experiments demonstrated the immunogenic S-II fraction had a buoyant density of 1.24 gm/cc in sucrose and 1.286 gm/cc in potassium bromide.

The particle size of the S-II fraction was estimated by electron microscopy and the distribution on the Sepharose 2B column. A predominate number of nearly spherical particles having diameters of 35-40 nm were evident in electron micrographs. A Stokes radius of 18 nm was estimated for the fraction based on its distribution coefficient on the column. Sedimentation velocity experiments with the fraction resulted in a single broad peak using Schlieren optics. A

sedimentation coefficient of approximately 100 Svedberg units was calculated for this peak. A particle weight of 7.9×10^6 was estimated for the S-II fraction based on its Stokes radius and sedimentation coefficient.

Serological studies of the concentrated fraction in gel diffusion plates using rabbit antiserum prepared by injecting rabbits with either the original culture filtrate or the 105,000 x g sediments resulted in two precipitin lines. The slower diffusing component appears to be an aggregate of the S-II immunogenic complex.

In conclusion gel filtration experiments and subsequent physical and chemical studies demonstrate that the major immunogenic substance isolated from cultures of avirulent P. multocida P-1059 Gray is a large toxic complex composed of lipopolysaccharide, protein and lipid. This complex was found to be relatively homogenous in terms of its particle size and composition.

LITERATURE CITED

Ackers, G. K. 1964. Molecular exclusion and restricted diffusion processes in molecular-sieve chromatography. Biochemistry 3:723.

Ackers, G. K., and Steere, R. L. 1967. Molecular sieve methods. In Mardmarosch, K. and Koprowski, H., Eds. Methods in virology. Vol. II. Pp. 325-365. New York, N.Y., Academic Press, Inc.

Adams, M. H. and Roe, A. S. 1945. A partially defined medium for cultivation of Pneumococcus. J. Bact. 49:401.

Andrews, P. 1970. Estimate of molecular size and molecular weights of biological compounds by gel filtration. In Glick, D., Ed. Methods of biochemical analysis. Vol. 18. Pp. 1-54. New York, N.Y., Interscience Publishers.

Ajl, S. J., Kadis, S. and Montie, T. C., Eds. 1970. Microbial toxins. Vol. I. Bacterial protein toxins. New York, N.Y., Academic Press, Inc.

Bailey, J. L. 1967. Techniques in protein chemistry. 2nd ed. New York, N.Y., Elsevier Publishing Co.

Bain, R.V.S. and Knox, K. W. 1961. Tha antigens of Pasteurella multocida on Type I. II. Lipopolysaccharides. Immunology 4:122.

Baxi, K. K., Blobel, H. and Scharmann, W. 1970. Untersuchungen über die Antigenstrucktin Von Pasteurella multocida. Zbl. Vet. Med., B, 17:441.

Bleiweis, A. S. and Coleman, S. E. 1969. Improved separation of components of streptococcal cell walls by thin layer chromatography. Anal. Biochem. 29:343.

Block, R. S., Durrum, E., and Zweig, G. 1958. Paper chromatography and paper electrophoresis. 2nd ed. New York, N.Y., Academic Press Inc.

Boivin, A. and Mesrobeanu, L. 1935. Recherches sur les Antigéns Somatiques et sur les Endotoxines des Bacteriés. I. Considerations Générales et Exposé des Techniques Utilisées. Rev. Immunol. 1:553.

Brown, J., Dawe, D. L., Davis, R. B., Foster, J. W., and Srivastava, K. K. 1970. Fowl cholera immunization in turkeys. I. Efficacy of various cell fractions of Pasteurella multocida as vaccines. Appl. Microbiol. 19:837.

Brückler, J., Baxi, K. K., and Blabel, H. 1970. Vertikale Polyacrylamidgel-Disk-Electrophorese von Pasteurella multocida-Proteinen. Zbl. Vet. Med. 17:564.

Cahn, R. D. 1967. Detergents in membrane filters. Science 115:195.

Cameron, J. A. 1968. Bacterial lipopolysaccharide as a void volume marker for agarose gel permeation chromatography. J. Chromatog. 37:331.

Carter, G. R. 1952. The type specific capsular antigen of Pasteurella multocida. Canad. J. Med. Sci. 30:48.

Carter, G. R. 1955. Studies on Pasteurella multocida I. A hemagglutination test for the identification of serological types. Amer. J. Vet. Res. 16:481.

Carter, G. R. and Annau, E. 1953. Isolation of capsular polysaccharides from colonial variants of Pasteurella multocida. Amer. J. Vet. Res. 14:475.

Carter, G. R. and Bain, R.V.S. 1960. Pasteurellosis (Pasteurella multocida). A review stressing recent developments. Vet. Reviews and Annotations 6, Part 2:106.

Carter, G. R. and Rappay, D. E. 1963. A hemagglutination test employing specific lipopolysaccharides for the detection and measurement of Pasteurella antibodies to Pasteurella multocida. Brit. Vet. J. 119:73.

Chervenka, C. H. 1969. A manual of methods for the analytical ulbacentrifuge. Palo Alto, Calif., Spinco Division, Beckman Instruments, Inc.

Cifonelli, J. A., Rebers, P. A. and Heddleston, K. H. 1970. The isolation and characterization of hyaluronic acid from Pasteurella multocida. Carbohyd. Res. 14:272.

Cox, D. J. and Schumaker, V. N. 1961. The perferential hydration of proteins in concentrated salt solutions. II. Sedimentation equilibrium of proteins in salt density gradients. J. Am. Chem. Soc. 83:2439.

Crutchley, M. J., Marsh, D. G. and Cameron, J. 1967. Free endotoxin. Nature 214:1052.

Crutchley, M. J., Marsh, D. G. and Cameron, J. 1968. Biological studies on free endotoxin and a non-toxic material from culture supernatant fluids of Escherichia coli 078K80. J. Gen. Microbiol. 50:413.

Dandeu, J. P. 1971. Chemical and immunological study of colicins E, K, A, and Q. Infection and Immunity 3:1.

Davis, R. B., Brown, J., Dawe, D. L., Foster, J. W., and K. K. Srivastava. 1970. Fowl cholera immunization in turkeys. III. Significance of market quanlity in the evaluation of fowl cholera vaccines. Appl. Microbiol. 19:844.

Dawe, D. L., Davis, R. B., Brown, J., Foster, J. W., and Srivastava, K. K. 1970. Fowl cholera immunization in turkeys. II. Use of experimental epornitic method to study vaccine efficacy in flocks of turkeys. Appl. Microbiol. 19:842.

Dhanda, M. R. 1960. Studies in immunochemistry. I. Isolation and characterization of the soluble antigens of Pasteurella septica. Indian J. Vet. Sci. 30:114.

Dische, Z. 1955. New color reactions for the determination of sugars in polysaccharides. In Glick, D., Ed. Methods of biochemical analysis. Vol. II. Pp. 313-358. New York, N.Y., Interscience Publishers, Inc.

Dittmer, J. C., and Lester, R. L. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. J. Lipid Res. 5:126.

Dubois, M, K., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350.

Dryer, R. L., Tammes, A. R., and Routh, J. I. 1957. The determination of phosphorus and phosphatase with N-phenyl-p-phenylenediamine. J. Biol. Chem. 225:177.

Ganfield, D. J., Rebers, P. A., and Heddleston, K. L. 1970. Gel filtration and isoelectric focusing of endotoxic-like antigens of Pasteurella multocida (Abstract). Fed. Proc. 29:637.

Gelotte, B. 1960. Studies on gel filtration. Sorption properties of the bed material Sephadex. J. Chromatog. 3:330.

Grabar, P. and Williams, C. A. 1953. Methode permetlant l' étude conjuguée des properiétés électrophorétiques et immunochimiques d'un mélange de protéins; application au sérum sanguin. Biochim. Biophys. Acta 10:193.

Groves, W. E., Davis, F. C., Jr., and Sells, B. H. 1968. Spectrophotometric determination of microgram quantities of protein without nucleic acid interference. Anal. Biochem. 22:195.

Hadley, P. 1918. Studies on fowl cholera. V. The toxins of Bacillus avisepticus. J. Bact. 3:277.

Harshfield, G. S. 1965. Fowl cholera. In Biester, H. E. and Schwarte, L. H., Eds. Diseases of poultry. 5th ed. Ames, Iowa, The Iowa State University Press.

Heddleston, K. L. 1962. Studies on pasteurellosis. V. Two immunogenic types of Pasteurella multocida associated with fowl cholera. Avian Dis. 6:315.

Heddleston, K. L. 1971. Avian pasteurellosis. In Biester, H. E. and Schwarte, L. H., Eds. Diseases of poultry. 6th ed. Chapter 5. Ames, Iowa, The Iowa State University Press (in press).

Heddleston, K. L., Gallagher, J. E. and Rebers, P. A. 1970. Fowl cholera: Immune response in turkeys. Avian Dis. 14:626.

Heddleston, K. L. and Hall, W. J. 1958. Studies on pasteurellosis. II. Comparative efficiency of killed vaccines against fowl cholera in chickens. Avian Dis. 2:322.

Heddleston, K. L. and Rebers, P. A. 1968. Fowl cholera: Active immunity induced in chickens and turkeys by oral administration of killed Pasteurella multocida. Avian Dis. 11:129.

Heddleston, K. L. and Rebers, P. A. 1969. Pasteurella multocida: Immune response in chicks and mice. U.S. Animal Health Assoc. Annual Meeting Proc. 73:280.

Heddleston, K. L., Rebers, P. A. and Ritchie, A. E. 1966. Immunizing and toxic properties of particulate antigens from two immunogenic types of Pasteurella multocida of Avian origin. J. of Immunol. 96:124.

Heddleston, K. L., Rhoades, K. R. and Rebers, P. A. 1967. Experimental Pasteurollosis: Comparative studies on Pasteurella multocida from Asia, Africa, and North America. Amer. J. Vet. Res. 28:1003.

Heddleston, K. L., Watko, L. P. and Rebers, P. A. 1964. Dissociation of a fowl cholera strain of Pasteurella multocida. Avian Dis. 8:649.

Homma, J. Y. and Suzuki, N. 1966. The protein moiety of the endotoxin of Pseudomonas aeruginosa. N.Y. Acad. Sci. Ann. 133:508.

Horsfall, F. L., Jr. and Tamm, I., Eds. 1965. Viral and rickettsial infections of man. 4th ed. Philadelphia, Pa., J. B. Lippincott Co.

Ifft, J. B. 1969. Proteins at sedimentation equilibrium in density gradients. In Alexander, P. and Lundgren, H. P., Eds. A laboratory manual of analytical methods of protein chemistry. Vol. 5. Pp. 153-223. New York, Pergamon Press.

Ifft, J. B. and Vinograd, J. 1966. The buoyant density of bovine serum mercaptalbumins in salt solutions at equilibrium in the ultracentrifuge. II. Net hydration, ion binding, and solvated molecular weights in various salt solutions. J. Phys. Chem. 70:2814.

Jenkin, C. R. and Rowley, D. 1959. Toxic proteins from Vibrio cholerae and water Vibrios which are lethal for mice. J. Gen. Microbiol. 21:191.

Kabat, E. A. and Mayer, M. 1961. Experimental immunochemistry 2nd ed. Chapter 13. Springfield, Illinois, Charles C. Thomas Publisher.

Kasai, N. 1966. Chemical studies on the lipid component of endotoxin, with special emphasis on its relation to biological activities. N.Y. Acad. Sci. Ann. 133:486.

Knox, K. W. and Bain, R.V.S. 1960. The antigens of Pasteurella multocida type 1. I. Capsular polysaccharides. Immunology 3:352.

Knox, K. W., Cullen, J., and Work, E. 1967. An extracellular lipopolysaccharide-phospholipid-protein complex produced by Escherichia coli grown under lysine-limiting conditions. Biochem. J. 103:192.

Knox, K. W., Vesk, M., and Work, E. 1966. Relation between excreted lipopolysaccharide complexes and surface structures of a lysine limited culture of Escherichia coli. J. Bact. 92:1206.

Kyaw, M. H. 1944. Pathogenesis of Pasteurella septica infection in developing chick embryo. J. Comp. Path. 54:200.

Kraeger, S. J. and Hamilton, J. G. 1969. Quantitative glass-paper chromatography of fungal cell wall acid hydrolysates. J. Chromotog. 41:113.

Landy, M. and Braun, W., Eds. 1964. Bacterial endotoxins. New Brunswick, N.J., Rutgers Univ. Press.

Langpap, T. J. and Matischeck, P. H. 1970. Observations on the prevalence of avian Pasteurella multocida serotypes. Avian Dis. 14:491.

Laurent, T. C. and Killander, J. 1964. A theory of gel filtration and its experimental verification. J. Chromatog. 14:317.

Little, P. A. and Lyon, B. M. 1943. Demonstration of serological types within the nonhemolytic Pasteurella. Am. J. Vet. Res. 4:110.

Lüderitz, O., Jann, K., and Wheat, R. 1968. Somatic and capsular antigens of Gram-negative bacteria. In Florkin, M. and Stotz, E. Comprehensive biochemistry. Vol. 26. Part A. Pp. 105-228. New York, N.Y., Elsevier Publishing Co.

Lüderitz, O., Staub, A. M., and Westphal, O. 1966. Immunochemistry of O and R antigens of Salmonella and related Enterobactericeae. Bact. Rev. 30:192.

MacLennan, A. P. and Davies, D.A.L. 1957. The isolation of D-glycero-D galacto heptore and other sugar components from the specific polysaccharide of Chromobacterium violaceum (BN). Biochem. J. 56:562.

MacLennan, A. P. and Rondle, C.J.M. 1957. Antigens of Pasteurella septica. J. Gen. Microbiol. 17:vii.

Marsh, D. G. and Crutchley, M. J. 1967. Purification and physicochemical analysis of fractions from the culture supernatant of E. coli. 0.78K80: Free endotoxin and a non-toxic fraction. J. Gen. Microbiol. 47:405.

Marsh, D. G. and Walker, P. D. 1968. Free endotoxin and non-toxic material from Gram-negative bacteria: Electron microscopy of fractions from E. coli. J. Gen. Microbiol. 52:125.

McIntire, F. C., Sievert, H. W., Barlow, G. H., Finley, R. A., and Lee, A. Y. 1967. Chemical, physical, and biological properties of a lipopolysaccharide from E. coli K-235. Biochemistry 6:2363.

Mergenhagen, S. E., Bladen, H. A., and Hsu, K. C. 1966. Election microscopic localization of endotoxic lipopoly-saccharide in gram-negative organisms. N.Y. Acad. Sci. Ann. 133:279.

Mesrobeanu, L., Mesrobeanu, I., and Mitrica, N. 1966. The neurotoxins of Gram-negative bacteria: The thermolabile endotoxin. N.Y. Acad. Sci. Ann. 133:685.

Montie, T. C. and Ajl, S. J. 1970. Nature and synthesis of marine toxins of Pasteurella pestis. In Montie, T. C., Kadis, S., and Ajl, S., Eds. Microbial toxins Vol. III. Pp. 1-37. New York, N.Y. Academic Press.

Montie, T. C., Montie, D. B., and Ajl, S. J. 1966. A comparison of the characteristics of two murine-toxic proteins from Pasteurella pestis. Biochem. Biophys. Acta 130:406.

Murata, M., Horiuchi, T., and Namioka, [illegible]. 1964. Studies on the pathogenicity of Pasteurella multocida for mice and chickens on the basis of O-groups. Cornell Vet. 54:293.

Namioka, S. and Murata, M. 1964. Serological studies on Pasteurella multocida. V. Some epizootiological findings resulting from O angigenic analysis. Cornell Vet. 54:520.

Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. J. Lipid Res. 9:693.

Nowotony, A. 1969. Molecular aspects of endotoxic reactions. Bact. Reviews 33:72.

Osborn, M. J. 1963. Studies on the gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of Salmonella typhimurim. Nat. Acad. Sci. Proc. 50:499.

Ouchterlony, O. 1948. In vitro method of testing the toxin-producing capacity of diptheria bacteria. Acta Path. Microbiol. Scand. 25:186.

Pasteur, L. 1880a. Sur le choléra des poules; études des conditions de la non-récidive de la maladie et de quelques autres de ses caractères. Compt. Rend. Acad. Sci. 90:952, 1030. English Translations in Chemical News 42:321 and 43:5.

Pasteur, L. 1880b. De L'altenuation des virus du cholera des poules. Compt. Rend. Acad. Sci. 91:673.

Page, M. and Godin, C. 1970. Determination of the Stokes radius of native and succinylated glutamate dehydrogenase on Sepharose 4B. J. Chromatog. 50:66.

Peacock, A. C. and Dingman, C. W. 1968. Molecular weight estimation and separation of ribonucleic acids by electrophoresis in agarose-acrylamide composite gels. Biochemistry 7:668.

Perreau, P. and Petit, J. P. 1963. Antigene lipopolysidique de Pasteurella multocida type E. Rev. Elevage Med. Vet. Pays Trop. 16:5.

Phelps, R. A. and Putnam, F. 1960. Chemical composition and molecular parameters of purified plasma proteins. In Putnam, F., Ed. The plasma proteins. Vol. I. Pp. 143-178. New York, N.Y. Academic Press Inc.

Pirosky, I. 1938. Sur l'antigene glucidolipidique des Pasteurella. Compt. Rend. Soc. Biol. 127:98.

Priestley, F. W. 1936. Some properties of the capsule of Pasteurella septica. Brit. J. Exptl. Path. 17:374.

Prince, G. H. and Smith, J. E. 1966a. Antigenic studies on Pasteurella multocida using immunodiffusion techniques. I. Identification and nomenclature of the soluble antigens of a bovine haemorrhagic septicaemia strain. J. Comp. Path. 76:303.

Prince, G. H. and Smith, J. E. 1966b. Antigenic studies on Pasteurella multocida using immunodiffusion techniques. II. Relationships with other Gram-negative species. J. Comp. Path. 76:315.

Prince, G. H. and Smith, J. E. 1966c. Antigenic studies on Pasteurella multocida using immunodiffusion techniques. III. Relationships between strains of Pasteurella multocida. J. Comp. Path. 76:321.

Raynaud, M. and Alouf, J. E. 1970. Intracellular vs. extracellular toxins. In Ajl, S. J., Kadis, S., and Monite, T. C., Eds. Microbial toxins. Vol. I. Bacterial protein toxins. Pp. 67 -117. New York, N.Y., Academic Press, Inc.

Raynaud, M., Digeon, J., and Naucid, C. 1964. Studies on the endotoxin and antigens of a rough strain of Salmonella typhi. In Landy, M. and Braun, W., Eds. Bacterial endotoxins. Pp. 326-344. New Brunswick, N.J. Rutgers University Press.

Rebers, P. A., Heddleston, K. L., and Ganfield, D. J. 1965. Isolation and characterization of a toxic, particulate and protective antigenic fraction from Pasteurella multocida (Abstract). Fed. Proc. 24:698.

Rebers, P. A., Heddleston, K. L., and Rhoades, K. R. 1967. Isolation from Pasteurella multocida of a lipopolysaccharide antigen with immunizing and toxic properties. J. Bact. 93:7.

Ribi, E., Milner, K. C., and Perrine, T. D. 1959. Endotoxin and antigenic fraction from the cell wall of Salmonella enteritidis. Methods for separation and some biological activities. J. Immunol. 82:75.

Roberts, R. S. 1947. An immunological study of Pasteurella septica. J. Comp. Path. 57:261.

Roberts, R. S. 1966. Preparation of endotoxin. Nature 209: 80.

Rogers, D. 1971. Release of a lipopolysaccharide-protein complex from Escherichia coli A by warm water treatment. Biochem. Biophys. Acta 230:72.

Rogers, S. W., Gilleland, H. E., Jr., and Eagon, R. G. 1969. Characterization of a protein-polysaccharide complex released from cell walls of Pseudomonas aeruginosa by ethylene diamine tetra acetic acid. Cans. J. Microbiol. 15:743.

Romanowska, E. 1970. Sepharose gel filtration method of purification of lipopolysaccharides. Anal. Biochem. 33:383.

Rondle, C.J.M. and Morgan, W.T.J. 1955. The determination of glucosamine and galactosamine. Biochem. J. 61:586.

Rothfield, L. and Pearlman-Kothencz, M. 1969. Synthesis and assembly of bacterial membrane components. A lipopolysaccharide-phospholipid protein complex excreted by living bacteria. J. Mol. Biol. 44:477.

Rothfield, L., Takeshita, M., Pearlman, M., and Horne, R. W. 1966. Role of phospolipids in the enzymatic synthesis of the bacterial cell envelope. Fed. Proc. 25, Part 1:1495.

Salmon, D. E. 1882. Report of the Commissioner of Agriculture for the years 1881 and 1882. Washington, D.C., U.S. Government Printing Office.

Shands, J. W., Jr., Graham, J. A., and Nath, K. 1967. The morphological structure of isolated bacterial lipopolysaccharide. J. Mol. Biol. 25:15.

Smith, I. 1960. Chromatographic and electrophoretic techniques. New York, N.Y., Interscience Publishers, Inc.

Snyder, F. and Stephens, N. 1959. A simplified spectrophotometric determination of ester groups in lipids. Biochem. Biophys. Acta 34:244.

Spirin, A. S. and Gavrilova, L. P. 1969. The ribosome. New York, N.Y., Springer-Verlag.

Srivastava, K. K., Foster, J. W., Dawe, D. L., Brown, J., and Davis, R. B. 1970. Immunization of mice with components of Pasteurella multocida. Appl. Microbiol. 20:951.

Straus, R. E. and Wurm, M. 1958. A new staining procedure and a method of quantitation of serum lipoproteins separated by paper electrophoresis. Am. J. Clin. Pathol. 29:581.

Taylor, A., Knox, K. W., and Work, E. 1966. Chemical and biological properties of an extracellular lipopolysaccharide from Escherichia coli grown under lysine-limiting conditions. Biochem. J. 99:53.

Uriel, J. 1969. Méthode d'électrophorese dans des gels d'acrylamidagarose. Bull. Soc. Chim. Biol. 48:969.

Uriel, J. and Grabar, P. 1961. A new technique for direct detection of glycoprotein and polysaccharides after electrophoresis or immunoelectrophoresis in agar gel. Anal. Biochem. 2:80.

van Heyningen , W. E. and Gladstone, G. P. 1953. The neurotoxin of Shigella shigae. I. Production, purification and properties of the toxin. Brit. J. Exptl. Pathol. 34:202.

Wang, W. S., Korczynski, M. S. and Lundgren, D. G. 1970. Cell envelope of an iron-oxidizing bacterium: Studies of lipopolysaccharide and peptidoglycan. J. Bact. 104: 556.

Weiser, M. M. and Rothfield, L. 1968. The reassociation of lipopolysaccharide, phospholipid, and transferase enzymes of the bacterial cell envelope. J. Biol. Chem. 243:1320.

Werner, M. J. 1966. Fractionation of lipoproteins from blood by gel filtration. J. Chromatog. 25:63.

Westphal, O., Lüderitz, O., and F. Bister. 1952. Über die Extraktion von Bakteriers mit Phenol/Wasser. Z. Naturfahn. 7b:148.

Wieme, R. J. 1965. Agar gel electrophoresis. New York, N.Y., Elsevier Publishing Co.

Yaw, K. Y., Briefman, L., and Kakavas, J. C. 1956. A comparison of virulence for mice and chickens of different colonial variants of the three serological types of Pasteurella multocida. Am. J. Vet. Res. 17:157.

Yaw, K. Y. and Kakavas, J. C. 1957. A comparison of the protection-inducing factors in chickens and mice of a type 1 strain of Pasteurella multocida. Am. J. Vet. Res. 18:661.

Zacharius, R. M., Zell, T. E., Manison, J. A., and Woodlock, J. J. 1969. Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem. 30:148.

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